

## PATENT COOPERATION TREATY

PCT

NOTIFICATION CONCERNING  
AMENDMENTS OF THE CLAIMS(PCT Rule 62 and  
Administrative Instructions, Section 417)

From the INTERNATIONAL BUREAU

To:

United States Patent and Trademark  
Office  
(Box PCT)  
Crystal Plaza 2  
Washington, DC 20231  
ETATS-UNIS D'AMERIQUE

in its capacity as International Preliminary Examining Authority

Date of mailing:

10 April 1997 (10.04.97)

International application No.:

PCT/IB96/01021

International filing date:

30 August 1996 (30.08.96)

Applicant:

RAMOT UNIVERSITY AUTHORITY FOR APPLIED RESEARCH AND INDUSTRIAL DEVELOPMENT  
LTD et al

The International Bureau hereby informs the International Preliminary Examining Authority that no amendments under Article 19 have been received by the International Bureau (Administrative Instructions, Section 417)

The International Bureau of WIPO  
34, chemin des Colombettes  
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorised officer:

H. Zhou

Telephone No.: (41-22) 730.91.11

## PATENT COOPERATION TREATY

PCT

## NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

United States Patent and Trademark  
Office  
(Box PCT)  
Crystal Plaza 2  
Washington, DC 20231  
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

<b>Date of mailing (day/month/year)</b> 10 April 1997 (10.04.97)	
<b>International application No.</b> PCT/IB96/01021	<b>Applicant's or agent's file reference</b> 2290.00037
<b>International filing date (day/month/year)</b> 30 August 1996 (30.08.96)	<b>Priority date (day/month/year)</b> 01 September 1995 (01.09.95)
<b>Applicant</b> LAVI, Sara	

1. The designated Office is hereby notified of its election made:



in the demand filed with the International Preliminary Examining Authority on:

13 March 1997 (13.03.97)



in a notice effecting later election filed with the International Bureau on:

2. The election ☒ was

was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO  
34, chemin des Colombettes  
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

H. Zhou

Telephone No.: (41-22) 730.91.11

# PATENT COOPERATION TREATY

PCT

## NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and  
Administrative Instructions, Section 422)

From the INTERNATIONAL BUREAU

To:

HACKMEY, Michal  
A.E. Mulford  
11 Shlom zion Hamalka Street  
P.O. Box 544  
91004 Jerusalem  
ISRAËL

<b>Date of mailing</b> (day/month/year) 06 November 1996 (06.11.96)	<b>IMPORTANT NOTIFICATION</b>
<b>Applicant's or agent's file reference</b> 2290.00037	
<b>International application No.</b> PCT/IB96/01021	<b>International filing date</b> (day/month/year) 30 August 1996 (30.08.96)

1. The following indications appeared on record concerning:

☐ the applicant ☐ the inventor ☐ the agent ☐ the common representative

Name and Address

Address for correspondence:  
KOHN, Kenneth, I.  
Kohn & Associates  
Suite 410  
30500 Northwestern Highway  
Farmington Hills, MI 48334  
United States of America

State of Nationality

State of Residence

Telephone No.

Facsimile No.

Teleprinter No.

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

☒ the person ☐ the name ☐ the address ☐ the nationality ☐ the residence

Name and Address

HACKMEY, Michal  
A.E. Mulford  
11 Shlom zion Hamalka Street  
P.O. Box 544  
91004 Jerusalem  
Israel

State of Nationality

State of Residence

Telephone No.

972-2-6231122/3

Facsimile No.

972-2-6246542

Teleprinter No.

3. Further observations, if necessary:

An agent has now been appointed.

4. A copy of this notification has been sent to:

☒ the receiving Office ☐ the designated Offices concerned  
☒ the International Searching Authority ☐ the elected Offices concerned  
☐ the International Preliminary Examining Authority ☒ other: KOHN, Kenneth, I.

The International Bureau of WIPO  
34, chemin des Colombettes  
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

F. Gateau

Telephone No.: (41-22) 730.91.11

## PATENT COOPERATION TREATY

PCT

COMMUNICATION OF  
INTERNATIONAL APPLICATIONS

(PCT Article 20)

From the INTERNATIONAL BUREAU

To:

United States Patent and Trademark  
Office  
(Box PCT)  
Crystal Plaza 2  
Washington, DC 20231  
ETATS-UNIS D'AMERIQUE

in its capacity as designated Office

Date of mailing:

19 June 1997 (19.06.97)

The International Bureau transmits herewith copies of the international applications having the following international application numbers and international publication numbers:

International application no.:

PCT/IB96/01021

International publication no.:

WO97/10796

**CORRECTED VERSION  
VERSION CORRIGEE**

The International Bureau of WIPO  
34, chemin des Colombettes  
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer:

J. Zahra  
Telephone No.: (41-22) 338.83.38



# PATENT COOPERATION TREATY

A. E. MULFORD

Received 30-11-1997

103

PCT NOTIFIED

From the  
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: MICHAL HACKMEY  
A. E. MULFORD, PATENT ATTORNEYS  
11 SHLOMZION HAMALKA ST.  
P.O. BOX 544  
JERUSALEM 91004  
ISRAEL

WRITTEN OPINION

(PCT Rule 66)

Response 12/10/97  
Out ASAP

Date of Mailing  
(day/month/year)

10 NOV 1997

REPLY DUE

within ONE month  
from the above date of mailing

Applicant's or agent's file reference

71714

International application No.

PCT/IB96/01021

International filing date (day/month/year)

30 AUGUST 1996

Priority date (day/month/year)

01 SEPTEMBER 1995

International Patent Classification (IPC) or both national classification and IPC  
Please See Supplemental Sheet.

Applicant

RAMOT-UNIVERSITY AUTHORITY FOR APPLIED RESEARCH AND INDUSTRIAL DEVELOPMENT LTD.

1. This written opinion is the first (first, etc.) drawn by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

- I ☒ Basis of the opinion
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step or industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☒ Certain observations on the international application

3. The applicant is hereby invited to reply to this opinion.

When? See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also For an additional opportunity to submit amendments, see Rule 66.4. For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 bis. For an informal communication with the examiner, see Rule 66.6. If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 01 JANUARY 1998

Name and mailing address of the IPHA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

JILL SCHMUCK

Telephone No. (703) 308-0196

JAB for

Form PCT/IPHA/408 (cover sheet) (January 1994)\*

WRITTEN OPINION

International application No.

PCT/IB96/01021

I. Basis of the opinion

1. This opinion has been drawn on the basis of (Substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed".):

☐ the international application as originally filed.

☒ the description, pages 1-79, as originally filed.  
pages NONE, filed with the demand.  
pages NONE, filed with the letter of \_\_\_\_\_

☒ the claims, Nos. 1-44, as originally filed.  
Nos. NONE, as amended under Article 19.  
Nos. NONE, filed with the demand.  
Nos. NONE, filed with the letter of \_\_\_\_\_

☒ the drawings, sheets/fig 1-6, 8-13, as originally filed.  
sheets/fig 7, filed with the demand.  
sheets/fig NONE, filed with the letter of \_\_\_\_\_

2. The amendments have resulted in the cancellation of:

☒ the description, pages NONE

☒ the claims, Nos. NONE

☒ the drawings, sheets/fig NONE

3. ☐ This opinion has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box Additional observations below (Rule 70.2(e)).

4. Additional observations, if necessary:  
NONE

WRITTEN OPINION

International application No.

PCT/IB96/01021

V. Reasoned statement under Rule 66.2(a)(II) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims <u>1-12, 15, 17, 20-39, 43-44</u>	YES
	Claims <u>13-14, 16, 18-19, 40-42</u>	NO
Inventive Step (IS)	Claims <u>1-12, 25-39</u>	YES
	Claims <u>13-24, 40-44</u>	NO
Industrial Applicability (IA)	Claims <u>1-44</u>	YES
	Claims <u>NONE</u>	NO

2. CITATIONS AND EXPLANATIONS

Claims 13-14, 16, 18 and 19 lack novelty under PCT Article 33(2) as being anticipated by Yokoyama et al. Yokoyama et al. disclose the expression of recombinant rat PP2C $\alpha$  phosphatase and the preparation of the polyclonal anti-PP2C phosphatase antibody. Thus, all of the elements taught by Yokoyama et al. meet all of the limitations of claims 13-16, 18 and 19.

Claims 40-42 lack novelty under PCT Article 33(2) as being anticipated by Schafer et al. Schafer et al. disclose that anti-FLAG M2 antibodies bind to the gene product of PP2C $\beta$ . Thus, all of the elements taught by Schafer et al. meet all of the limitations of claims 40-42.

Claims 15, 17 and 20-24 lack an inventive step under PCT Article 33(3) as being obvious over Yokoyama et al. Yokoyama et al. disclose the expression of recombinant rat PP2C $\alpha$  phosphatase and the preparation of the polyclonal anti-PP2C phosphatase antibody.

Accordingly, in view of the cited art teaching the isolation of cDNA encoding PP2C $\alpha$ , one of ordinary skill in the art would have been motivated to use the cDNA encoding PP2C $\alpha$  to construct expression constructs comprising PP2C $\alpha$  antisense sequences; to prepare various monoclonal and polyclonal antibodies; and to produce PP2C $\alpha$  peptides because such work is routine technology in the art with a reasonable expectation of producing functional PP2C $\alpha$  expression constructs, antibodies, and/or peptides.

Claims 43-44 lack an inventive step under PCT Article 33(3) as being obvious over Schafer et al. in view of Yokoyama et al.

Schafer et al. disclose the expression of recombinant MPP $\beta$ 4 protein.

Accordingly, in view of the cited art teaching the production of recombinant MPP $\beta$ 4 and the recombinant antibody technology taught by Yokoyama et al., one of ordinary skill in (Continued on Supplemental Sheet.)

WRITTEN OPINION

International application No.

PCT/IB96/01021

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

The description is objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 5 because it fails to adequately enable practice of the claimed invention because: the description fails to provide guidance or direction for the production of non-human transgenic animals or organisms of all species, as in claims 11-12, which express high enough levels of PP2C $\alpha$  or polymorphisms thereof to cause a useful phenotype which distinguishes the animal over a wild-type animal or organism. It is well known in the art of transgenics that species differences in the expression of a transgene make it unpredictable to expect high enough or identical levels of expression of a transgene in different species of animals as well as organisms.

Further, in regard to claims 1-10 and 25-39, the description fails to provide direction or guidance with respect to diagnosing a patient to have cancer and/or treating a patient with respect to predictably achieving targetability of an agent, high enough levels of transcription of an agent to effectively modulate expression of PP2C $\alpha$  in a patient. For example, the description fails to provide evidence that transcription of antisense in vivo results in the regulation of PP2C $\alpha$  expression such that treatment of all of the pathologies related to cancer is rendered. Additionally, the description fails to teach suitable combinations of promoters and antisense which effectively targets and results in high enough levels of transcription to render therapeutic results in patients. It is well known in the art that gene therapy is unpredictable and the skill in the art is high. Further, it is also well known that antisense therapy is even more unpredictable in regard to determining antisense sequences which effectively bind to the target.

WRITTEN OPINION

International application No.

PCT/IB96/01021

Supplemental Box  
(To be used when the space in any of the preceding boxes is not sufficient)

Sheet 10

Continuation of: Boxes I - VIII

**TIME LIMIT:**

The time limit set for response to a Written Opinion may not be extended. 37 CFR 1.484(d). Any response received after the expiration of the time limit set in the Written Opinion will not be considered in preparing the International Preliminary Examination Report.

**CLASSIFICATION:**

The International Patent Classification (IPC) and/or the National classification are as listed below:  
IPC(6): C12N 15/63, 5/00, 15/00; A01N 43/04; A61K 31/70 and US Cl.: 435/172.3, 69.1, 69.2, 325, 7.1, 91.2, 320.1; 530/350, 387.1; 800/2; 536/23.1, 23.5; 424/93.21, 9.2

**V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):**

the art would have been motivated to use the recombinant MPPβ4 to produce various monoclonal and polyclonal antibodies raised against the peptide with a reasonable expectation of producing functional MPPβ4 antibodies.

Claims 1-44 meet the criteria set out in PCT Article 33(4) because the claimed invention is useful for the study of PP2C gene activity and its relationship to the pathology of cancer and thus, the detection and treatment thereof.

**NEW CITATIONS**

NONE

## PATENT COOPERATION TREATY

## PCT

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

REC'D 24 FEB. 1998

WIPO PCT

Applicant's or agent's file reference 7/77/4	<b>FOR FURTHER ACTION</b> See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/IB96/01021	International filing date (day/month/year) 30 AUGUST 1996	Priority date (day/month/year) 01 SEPTEMBER 1995
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant RAMOT-UNIVERSITY AUTHORITY FOR APPLIED RESEARCH AND INDUSTRIAL DEVELOPMENT LTD.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets.

☒ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 1 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☒ Certain observations on the international application

Date of submission of the demand  13 MARCH 1997	Date of completion of this report  30 JANUARY 1998
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer  JILL SCHMUCK <i>[Signature]</i>
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/IB96/01021

**I. Basis of the report**

1. This report has been drawn on the basis of (*Substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments*):

☒ the international application as originally filed.

☒ the description, pages 1-79, as originally filed.

pages NONE, filed with the demand.

pages NONE, filed with the letter of \_\_\_\_\_.

pages \_\_\_\_\_, filed with the letter of \_\_\_\_\_.

☒ the claims, Nos. 1-44, as originally filed.

Nos. NONE, as amended under Article 19.

Nos. NONE, filed with the demand.

Nos. NONE, filed with the letter of \_\_\_\_\_.

Nos. \_\_\_\_\_, filed with the letter of \_\_\_\_\_.

☒ the drawings, sheets/~~fig~~ 1-6, 8-13, as originally filed.

sheets/~~fig~~ 7, filed with the demand.

sheets/~~fig~~ NONE, filed with the letter of \_\_\_\_\_.

sheets/~~fig~~ \_\_\_\_\_, filed with the letter of \_\_\_\_\_.

2. The amendments have resulted in the cancellation of:

☒ the description, pages NONE.

☒ the claims, Nos. NONE.

☒ the drawings, sheets/~~fig~~ NONE.

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the ~~Supplemental Box~~ Additional observations below (Rule 70.2(c)).

4. Additional observations, if necessary:

NONE

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/IB96/01021

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement****1. STATEMENT**

Novelty (N)	Claims	<u>1-12, 15, 17, 20-39, 43-44</u>	YES
	Claims	<u>13-14, 16, 18-19, 40-42</u>	NO
Inventive Step (IS)	Claims	<u>1-12, 25-39</u>	YES
	Claims	<u>13-24, 40-44</u>	NO
Industrial Applicability (IA)	Claims	<u>1-44</u>	YES
	Claims	<u>NONE</u>	NO

**2. CITATIONS AND EXPLANATIONS**

Claims 13-14, 16, 18 and 19 lacks novelty under PCT Article 33(2) as being anticipated by Yokoyama et al. Yokoyama et al. disclose the expression of recombinant rat PP2C $\alpha$  phosphatase and the preparation of the polyclonal anti-PP2C phosphatase antibody.

Thus, all of the elements taught by Yokoyama et al. meet all of the limitations of claims 13-16, 18 and 19.

Claims 40-42 lack novelty under PCT Article 33(2) as being anticipated by Schafer et al. Schafer et al. disclose that anti-FLAG M2 antibodies bind to the gene product of PP2C $\beta$ . Thus, all of the elements taught by Schafer et al. meet all of the limitations of claims 40-42.

Claims 15, 17 and 20-24 lack an inventive step under PCT Article 33(3) as being obvious over Yokoyama et al. Yokoyama et al. disclose the expression of recombinant rat PP2C $\alpha$  phosphatase and the preparation of the polyclonal anti-PP2C phosphatase antibody.

Accordingly, in view of the cited art teaching the isolation of cDNA encoding PP2C $\alpha$ , one of ordinary skill in the art would have been motivated to use the cDNA encoding PP2C $\alpha$  to construct expression constructs comprising PP2C $\alpha$  antisense sequences; to prepare various monoclonal and polyclonal antibodies; and to produce PP2C $\alpha$  peptides because such work is routine technology in the art with a reasonable expectation of producing functional PP2C $\alpha$  expression constructs, antibodies, and/or peptides.

Claims 43-44 lack an inventive step under PCT Article 33(3) as being obvious over Schafer et al. in view of Yokoyama et al.

Schafer et al. disclose the expression of recombinant MPP $\beta$ 4 protein.

Accordingly, in view of the cited art teaching the production of recombinant MPP $\beta$ 4 and the recombinant antibody technology taught by Yokoyama et al., one of ordinary skill in the art would have been motivated to use the recombinant MPP $\beta$ 4 to produce various monoclonal and polyclonal antibodies raised (Continued on Supplemental Sheet.)



**VIII. Certain observations on the international application**

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

The description is objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 5 because it fails to adequately enable practice of the claimed invention because: the description fails to provide guidance or direction for the production of non-human transgenic animals or organisms of all species, as in claims 11-12, which express high enough levels of PP2C $\alpha$  or polymorphisms thereof to cause a useful phenotype which distinguishes the animal over a wild-type animal or organism. It is well known in the art of transgenics that species differences in the expression of a transgene make it unpredictable to expect high enough or identical levels of expression of a transgene in different species of animals as well as organisms.

Further, in regard to claims 1-10 and 25-39, the description fails to provide direction or guidance with respect to diagnosing a patient to have cancer and/or treating a patient with respect to predictably achieving targetability of an agent, high enough levels of transcription of an agent to effectively modulate expression of PP2C $\alpha$  in a patient. For example, the description fails to provide evidence that transcription of antisense in vivo results in the regulation of PP2C $\alpha$  expression such that treatment of all of the pathologies related to cancer is rendered. Additionally, the description fails to teach suitable combinations of promoters and antisense which effectively targets and results in high enough levels of transcription to render therapeutic results in patients. It is well known in the art that gene therapy is unpredictable and the skill in the art is high. Further, it is also well known that antisense therapy is even more unpredictable in regard to determining antisense sequences which effectively bind to the target.

# INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/IB96/01021

## Supplemental Box

(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

Sheet 10

### CLASSIFICATION:

The International Patent Classification (IPC) and/or the National classification are as listed below:

IPC(6): C12N 15/63, 5/00, 15/00; A01N 43/04; A61K 31/70 and US Cl.: 435/172.3, 69.1, 69.2, 325, 7.1, 91.2, 320.1; 530/350, 387.1; 800/2; 536/23.1, 23.5; 424/93.21, 9.2

### V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):

against the peptide with a reasonable expectation of producing functional MPP $\beta$ 4 antibodies.

Claims 1-12 and 25-39 meet the criteria set out in PCT Articles 33(2) and 33(3), because the prior art does not teach or fairly suggest the claimed invention.

Claims 1-44 meet the criteria set out in PCT Article 33(4) because the claimed invention is useful for the study of PP2C gene activity and its relationship to the pathology of cancer and thus, the detection and treatment thereof.

----- NEW CITATIONS -----

NONE

PCT/IB 96/01021  
IPEA/US 13 MAR 1997

7/13

Fig-6a

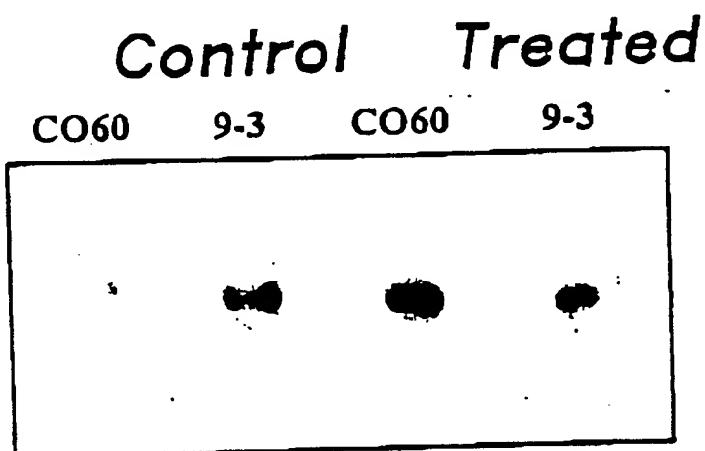


Fig-6b

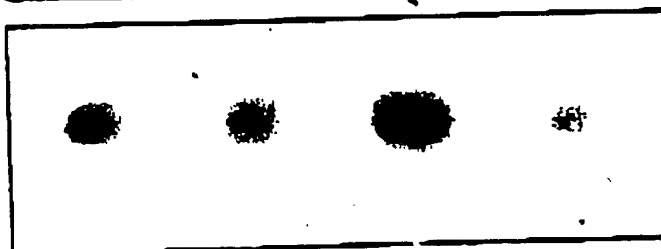


Fig-6c



**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>A61K</b>	<b>A2</b>	<b>(11) International Publication Number:</b> <b>WO 97/10796</b> <b>(43) International Publication Date:</b> 27 March 1997 (27.03.97)
<b>(21) International Application Number:</b> PCT/IB96/01021 <b>(22) International Filing Date:</b> 30 August 1996 (30.08.96) <b>(30) Priority Data:</b> 60/003,114 1 September 1995 (01.09.95) US <b>(71) Applicant (for all designated States except US):</b> RAMOT-UNIVERSITY AUTHORITY FOR APPLIED RESEARCH AND INDUSTRIAL DEVELOPMENT LTD. [IL/IL]; 32 Haim Levanon Street, Ramat-Aviv, 61392 Tel-Aviv (IL). <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only):</b> LAVI, Sara [IL/IL]; 6 Tnuat Hameri Street, 55000 Kyriat Ono (IL). <b>(74) Agent:</b> HACKMEY, Michal; A.E. Mulford, 11 Shlom zion Hamalka Street, P.O. Box 544, 91004 Jerusalem (IL).		<b>(81) Designated States:</b> AL, AU, BB, BG, BR, CA, CN, CU, CZ, EE, GE, HU, IL, IS, JP, KP, KR, LK, LR, LT, LV, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, TR, TT, UA, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> MANIPULATION AND DETECTION OF PROTEIN PHOSPHATASE 2C - PP2C $\alpha$ - EXPRESSION IN TUMOR CELLS FOR CANCER THERAPY, PREVENTION AND DETECTION		
<b>(57) Abstract</b>  A method of detecting cancer in a patient by detecting alteration of activity of the gene coding for human type protein phosphatase 2C (PP2C $\alpha$ and PP2C $\beta$ ) and genetic polymorphisms thereof in a specimen isolated from the patient is disclosed. The invention further provides a method of treating cancer including the steps of first determining the type of cancer and cells expressing the cancer and then preparing a vector which will specifically target the cancer cells and can include regulatory elements to control the expressibility of PP2C $\alpha$ . The vector is then administered to the patient. Alternatively an antisense vector can be prepared.		

MANIPULATION AND DETECTION OF PROTEIN  
PHOSPHATASE 2C - PP2C $\alpha$  - EXPRESSION IN TUMOR  
CELLS FOR CANCER THERAPY, PREVENTION AND  
DETECTION

5

BACKGROUND OF THE INVENTION

TECHNICAL FIELD

10 The present invention relates to detection and  
methods of treating cancer by utilizing the gene human  
type protein phosphatase 2C (PP2C $\alpha$  and PP2C $\beta$ ) and gene  
products thereof and kits for the practice of the  
invention; preparing native and transgenic organisms in  
15 which the gene products encoded by the human PP2C $\alpha$  gene  
or its homolog in other species are produced, or the  
expression of the native PP2C $\alpha$  gene is modified or  
knocked out.

20

BACKGROUND ART

Transformed or malignant cells pose a severe  
health threat. If the transformed phenotype can be  
reversed the cancerous cells can be controlled  
providing a treatment. In reversal the cells can loose  
25 their neoplastic phenotype thereby reinstating normal  
cellular growth and/or differentiation, or there is  
growth arrest or there can be activation of programmed  
cell death - apoptosis pathway. It would be useful to  
provide therapeutic measures which can reverse the  
transformed phenotype by instituting any of these  
30 reversal means by therapeutic measures. Further, early  
identification of transformation events would also  
prove useful, since therapy could be initiated sooner.

Genes have now been identified that are involved  
35 in transformation such as Ras, Fos PDGF, erb-B, erb-B2,  
RET, c-myc, Bcl-2, APC, NF-1, RB, p53, etc. The genes

fall into two broad categories proto-oncogenes and tumor suppressor genes. Proto-oncogenes code for proteins that stimulate cell division and when mutated (oncogenes) cause stimulatory proteins to be overactive with the result that cells over proliferate. Tumor suppressor genes code for proteins that suppress cell division. Mutations and/or aberrant regulation can cause these proteins to be inactivated thereby rendering the cells without proliferation restraint. Additionally, E2F and p53 and others can act as both oncogene and tumor suppressor gene when improperly expressed. Among the oncogenes and tumor suppressor genes are motifs which act as transcription factors and as protein kinase. The identification of these specific genes have disclosed some of how the cell life cycle progresses.

Gene amplification is one of the distinct abnormalities associated with malignant cells and transformed cell lines [see generally "Gene Amplification in Mammalian Cells, A comprehensive Guide. edited by R. E. Hellems, Marcel Dekker, Inc. for a review of amplification.] This phenomenon is part of the genetic instability characterizing neoplastic cells and occurs rarely in normal cells. Some oncogenes and tumor suppressor genes have been shown to be amplified such as Ras, Erb, p53 etc.

Phosphorylation of structural and regulatory proteins including oncogenes and tumor suppressor genes is a major intracellular control mechanism in eukaryotes [Wera and Hemmings, 1995; Cohen, 1989]. Protein phosphorylation and dephosphorylation is part of the regulatory cycle for signal transduction, cell cycle progression and transcriptional control. Protein kinases and protein phosphatases both have roles in the phosphorylation - dephosphorylation cycle, respectively. Mutations in the genes coding for these

proteins can lead to failure of protein phosphorylation. For example, in yeast, mutations of a type 2C protein phosphatases lead to a defect in osmoregulation [Shiozaki and Russell, 1995].

5       pp2c is a protein serine/threonine phosphatase [Cohen 1989]. The pp2c family consists of two cytoplasmic isoenzymes in mammalian tissues [McGowan and Cohen, 1987] and at least three pp2c-like enzymes in yeast show the same enzymatic and biochemical  
10       properties. The two mammalian isoenzymes are monomers but differ slightly in molecular mass (44KDa and 42KDa) and are designated pp2c $\alpha$  and pp2c $\beta$ . There is conflicting literature as to their function and association of these protein phosphatases with  
15       transformed cells [Saadat et al, 1994; Nishikawa, et al, 1995; Lau and Baylink, 1993; Shiozaki et al, 1994; Eden and Cedar, 1994; McGowan and Cohen, 1987; Wenk and Mieskes, 1995]

20       It would be useful to be able to therapeutically control protein phosphorylation where needed for normal cell function. Additionally, glycosylation following mRNA translation is essential for the functioning of many gene products. Aberrant glycosylation of proteins can interfere with protein function and can result from  
25       an altered regulatory pathway. An important mode of control of gene expression is DNA methylation [Eden and Cedar, 1994]. Aberrant methylation of DNA can play a role and lead to improper expression of regulatory proteins controlling cell cycle.

30       Viruses are very specialized infectious agents that have evolved, in many cases, to elude host defense mechanisms. Adeno associated viruses are members of the family of parvoviruses for which tumor suppressive properties have already been described in 1960 [for  
35       review see Rommelaere and Tattersal, 1990]. They are a group of small viruses, with a ssDNA genome of

approximately 5000 nucleotides, characterized by identical palindromic termini of 154 bases. The left part of the AAVDA3 genome encodes four multifunctional, overlapping, non-structural proteins (Rep78, Rep68, Rep52 and Rep40) which are translated from differentially spliced mRNA driven by the P5 and P19 promoters (Accession numbers J01901, M12405, M12468, M12469). In the right part of the genome three overlapping capsid polypeptides (VP1 - VP3) are encoded from the P40 promoter [Berns, 1990; Leonard and Berns, 1994]. These extremely small DNA viruses are represented in vertebrates by two genera, the autonomously replicating and the helper dependent parvovirus [Siegl et al., 1985].

The helper-dependent adeno-associated viruses (AAV) depend for their replication on coinfecting helper virus [Young and Mayor, 1979a,b], or on conditions of genotoxic stress [Yakobson et al., 1987] and comprise agents infecting humans without apparent disease [Cukor et al., 1984]. Helper viruses are adenoviruses [Atchison et al., 1965], herpes group viruses [Salo and Mayor, 1979] and vaccinia virus [Schlehofer et al., 1986]. The helper viruses share the ability to induce chromosomal damage early in their infection cycle [Schlehofer and zur Hausen, 1982].

Tumor suppressive properties have been found for AAV [for review see Schlehofer, 1994]. It has been shown that the development of tumors induced in rodents by adenoviruses, herpes viruses or by transplantation of cells transformed by these viruses could be inhibited by infecting the animal cells with AAV [Kirschstein et al. 1968; Mayor et al., 1973; de la Maza and Carter, 1981; Ostrove et al., 1981]. The *in vivo* findings of tumor suppression are paralleled by results showing inhibition of cellular transformation *in vitro*. This could be shown for cells of different origin



(hamster and mouse) transformed by viruses or by activated oncogenes. Compared with controls, cells infected with AAV or transfected with specific AAV DNA sequences displayed decreased focus formation and saturation density indicating inhibition of transformation-associated traits [Casto and Goodheart, 1972; Katz and Carter, 1986; Hermonat, 1989; Hermonat, 1994; Schlehofer et al, 1983; Schlehofer, 1994; Yang et al, 1995; Kleinschmidt et al, 1995].

In addition, there are seroepidemiologic findings in the human population, showing that cancer patients exhibit antibodies to AAV less frequently than matched control individuals. Three independent studies carried out in the USA [Mayor et al., 1976], Belgium [Sprecher-Goldberger et al., 1971] and Germany [Georg-Fries et al., 1984] using different serologic techniques, have found a high prevalence of antibodies to AAV (types 2, 3, and 5) in the normal population contrasting with a relatively low frequency of seropositivity in patients with cancer.

It would be useful to develop therapeutic methods for controlling cell transformation. As the above information indicates, it is possible to reverse cell transformation or to specifically kill the transformed cell. Given the available anti-sense technology, vector delivery technology and the like it would be useful to find human cellular mechanisms that can be controlled to reverse cell transformation with these methods or others as they become known.

#### SUMMARY OF THE INVENTION

According to the present invention, a method and kit of detecting cancer in a patient by detecting alterations of the activity of the gene (PP2C $\alpha$  or PP2C $\beta$ ) coding for human type protein phosphatase 2C

(pp2c $\alpha$ ) and genetic polymorphisms thereof in a specimen isolated from the patient is disclosed.

The invention further provides a method of treating cancer including the steps of first  
5 determining the type of cancer and cells expressing the cancer and then preparing a vector which will specifically target the cancer cells and can include regulatory elements to control the expressibility of PP2C $\alpha$ . The vector is then administered to the patient.  
10 Alternatively an antisense vector can be prepared.

The invention further provides a method of treating diseases due to aberrant phosphorylation due to alteration of expression of PP2C $\alpha$  by controlling PP2C $\alpha$  expression.  
15

#### BRIEF DESCRIPTION OF THE DRAWINGS

Other advantages of the present invention will be readily appreciated as the same becomes better  
20 understood by reference to the following detailed description when considered in connection with the accompanying drawings wherein:

FIGURE 1A-B are graphs of a FACS analysis of CO60 and two AAV/neo cell lines 913 and 916 as prepared for  
25 cell cycle analysis. 24 hours after seeding the cell were trypsinized and washed with PBS. The cells were resuspended in 1ml buffer containing 0.1% sodium citrate, 0.1% triton X-100 and 50  $\mu$ g propidium iodide, and then processed in the FACS.

FIGURE 2 is a photograph of a Southern Blot  
30 Analysis showing CHINT is associated with AAV integration in different AAV/neo cell lines. Southern blot analysis of different AAV/neo clones, CO60 DNA, digested with BglIII, and hybridized with "CHINT" probe.  
35 9-1, 2, 3, 4 and 5 on AAV/neo cell lines. 93R is a

-7-

revertant that lost the whole chromosome containing the AAV. A6 is a mouse cell line.

FIGURE 3 is a schematic representation of the organization of the integrated AAV and the flanking cellular sequences in 9-3 cells. A genomic library was prepared from C9-3 cells using the EMBL-4 lambda phage and scored for AAV positive clones. A clone of 13Kb- $\lambda$ SL9-1 was isolated and later subcloned to a blue-script vector. Plasmids pSL9-11 (13Kb), pSL9-8 (10Kb) and pSL9-6 (3Kb) were obtained as indicated in the figure.

FIGURE 4A-B wherein (A) is a photograph of a Southern Blot Analysis showing AAV is adjacent to the gene coding to PP2C $\alpha$  in 9-3 cells. The Southern blot analysis of genomic DNA, from CO60 and 9-3 cells digested by *EcoRI*, or *XbaI* was hybridized sequentially with the following probes: 1) AAV; 2) CHINT; and 3) Rat PP2C $\alpha$  probes. The CHINT and the PP2C $\alpha$  sequences are adjacent (4Kb *EcoRI* fragment). The AAV CHINT and PP2C $\alpha$  are in a close proximity in 9-3 cells (the 5.6Kb *XbaI* fragment). (B) pSL9-6 is adjacent to PP2C $\alpha$  in the wild type Chinese hamster cells. *BamHI* digested DNA from the Chinese hamster neo cells was hybridized to pSL9-6 and PP2C $\alpha$  probes. A common fragment of ~8.5Kb appeared in all cell lines including CO60. The same fragment hybridized also to CHINT probe (data not shown).

FIGURE 5A-C are photographs of a Southern blot analysis of DA3 (lane 8) and DA3J1-DA3J7 cells lines (lanes 1-7). Genomic DNA was digested with *BglIII*. The blots were hybridized sequentially with an AAV/neo JDT277, pSL9-6 and PP2C $\alpha$  PCR probes. A 4Kb fragment hybridized to the AAV probe and pSL9-6 probe in J3 (lane 3), J4 (lane 4) and J6 (lane 6). A fragment smaller than 4Kb hybridized to both AAV and PP2C $\alpha$  probe in J1 (lane 1), J2 (lane 2), J5 (lane 5) and J6 (lane 6).

FIGURE 6 is a photograph which shows the alteration in PP2C $\alpha$  mRNA in response to carcinogen treatment. Forty  $\mu$ g of total RNA were isolated from CO60 and C9-3 cells 48 hours after treatment with MNNG (7.5  $\mu$ g/ml and 2.5  $\mu$ g/ml respectively), and from untreated cells and fractionated on a denaturing gel (1.2% agarose/6.6% formaldehyde gel). The gel was blotted and hybridized consecutively with  $^{32}$ P-labeled rat PP2C $\alpha$  cDNA (A) pSL9-1<sup>DNA</sup> (B) and rRNA cDNA (C).

FIGURE 7 is a photograph which shows gel electrophoresis analysis after 25, 30 and 35 PCR cycles. The oligo dT-primed cDNA obtained from colorectal tumor No. 6 (T6), or from its adjacent nontumorous mucosa (N6), were subjected to PCR reactions using the specific PP2C $\alpha$  and the  $\beta$ -actin sense and anti-sense primers. The 25 cycle PCR cycle for PP2C $\alpha$  cDNA in the normal and tumor tissues is not shown since a visible product was not found.

FIGURE 8 is a photograph which shows gel electrophoresis analysis after 25, 30, 30 and 35 PCR cycles of aliquots of the oligo dT-primed cDNA obtained from CHE cell line, or from its adjacent transformed cell line (CO60), subjected to PCR reactions using the specific PP2C $\alpha$  and the  $\beta$ -actin sense and anti-sense primers.

FIGURE 9A-B are schematic representations of plasmids that contain PP2C $\alpha$  cDNA in the (A) sense orientation (pYM001) and in the (B) antisense orientation (pYM002).

FIGURE 10 is a photograph which shows gel electrophoresis analysis of immunoprecipitation of liver extracts with a panel of monoclonal antibodies raised against pp2c $\alpha$ ; 1D5, 2A3, 9F4, 9F1, are monoclonal antibodies used to precipitate pp2c $\alpha$  from liver extract; 801 and 351 are rabbit polyclonal antibodies used for detection after immunoblotting.

FIGURE 11 is a schematic representation of a genomic  $\lambda$ 100 clone containing the first translated exon of PP2C $\alpha$ . The phage was cloned from a CHO library. The sequenced regions are indicated by cross hatching  
5 (SEQ ID Nos:15 and 16).

FIGURE 12 is a photograph which shows gel electrophoresis wherein lane 1: Cotransfection with pSK1 and pAV2; lane 2: Transfection with the SV40 plasmid pSK1 SV40 replicates; lane 3: Cotransfection of  
10 pSVK1 and a plasmid harboring 140bp from the AAV genome nucleotide 125-263; lane 4: Cotransfection of pSVK1 with pSL9-6.

FIGURE 13 is a photograph of a Northern blot wherein RNA from various mouse tissues is hybridized  
15 with PP2C $\alpha$  cDNA demonstrating that there are several mRNAs of different sizes ranging from less than 2 kb to higher than 5.0kb. RNA was extracted from ovary (O), Testis (T), Kidney (K), Liver (L), Muscle (M), Heart (H), Lung (Lu) and Brain (B).

20

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention discloses a method of  
25 detecting cancer in a patient by detecting alterations in gene activity of the gene (PP2C $\alpha$ ) coding for human type protein phosphatase 2C (pp2c $\alpha$ ) and genetic polymorphisms thereof in a specimen isolated from the patient. The gene activity of the patient is compared  
30 to that of normal controls. Alterations in activity can be a down-regulation of the gene activity or conversely an up-regulation resulting in changes in phosphorylation. Further, alterations can result in aberrant function or absence of the gene product and in  
35 a change in distribution of the gene product within the cell itself.

Polymorphisms are variants in the gene sequence. They can be sequence shifts found between different ethnic and geographic locations which, while having a different sequence, produce functionally equivalent gene products, isoforms. Polymorphisms also encompass variations which can be classified as alleles and/or mutations which can produce gene products which may have an altered function. Polymorphisms also encompass variations which can be classified as alleles and/or mutations which either produce no gene product, an inactive gene product or increased levels of gene product. Polymorphisms as used herein can also encompass variations which are due to differences in DNA methylation in control and coding regions. Further, the term is also used interchangeably with allele as appropriate.

Cancer is defined as transformed or malignant cells, i.e. cells undergoing uncontrolled growth and spread (see generally, Scientific American September, 1996 for a review).

In general, it is found in cell transformation as shown in the Examples herein below (Examples 4, 5) that the activity/expression of PP2C $\alpha$  is reduced compared to that of normal controls as determined by a reduction in the amount of gene product in the cell. However, it is contemplated by the present invention that increased activity results in changes in protein activity such that there are changes in cell function.

Further it is recognized by the present invention that there is more than one form of the gene product of PP2C $\alpha$  and that one may be reduced or altered in cells while another specific form of PP2C $\alpha$  will be elevated or more prominent compared to normal controls. The cells can be any cell type that shows alteration in PP2C $\alpha$  activity in a disease state. Further, a second gene may be controlled by the alteration in the

activity of PP2C $\alpha$  such that their products are elevated or reduced and can be monitored by the method of the present invention. New transcripts, absence of transcripts or alterations in the protein coded by these transcripts are monitored.

Further, the present invention recognizes that pp2c $\alpha$  is itself phosphorylated as it has several phosphorylation sites including tyrosine, serine and threonine and that failure to phosphorylate it properly will cause malfunction of the pp2c $\alpha$  protein. Further, pp2c $\alpha$  also dephosphorylates itself. A failure in its autophosphorylation will have effects on cell cycle regulation.

Samples can be biopsied material from suspected precancerous lesions or any tissue or bodily fluid which can be assayed for PP2C $\alpha$  activity or gene product as described herein. Bodily fluids such as blood, urine, cerebrospinal fluid and saliva can be examined as is appropriate.

In an embodiment the detection of PP2C $\alpha$  activity is by assaying the specimen for mRNA complementary to PP2C $\alpha$  DNA including polymorphisms thereof with an assay selected from the group consisting of *in situ* hybridization, Northern blotting and reverse transcriptase - polymerase chain reaction.

In an alternative method, the detecting of PP2C $\alpha$  activity and cellular distribution is by assaying the specimen for a PP2C $\alpha$  gene product including polymorphisms and peptide fragments thereof with an assay selected from the group consisting immunohistochemical and immunocytochemical staining, ELISA, RIA, immunoblots, immunoprecipitation, Western blotting, functional assays for activity of gene product, assays for phosphorylation patterns and protein truncation test. Target proteins which are dephosphorylated by pp2c $\alpha$  can have different size

characteristics on PAGE and different isoelectric points as well as changes in function such as their ability to interact with other proteins, RNA, DNA and other cellular components.

5 Further, if chromosomal abnormalities are associated with altered PP2C $\alpha$  these are screened for using standard methods known in the art.

In addition to changes in the location and amount of the gene product in the cell itself as shown in the  
10 Examples herein below, the method of the present invention screens for the gene product in bodily fluids. With alteration in gene function the level of gene product in the bodily fluid is affected as for example more can be released from the cell if  
15 glycosylation or signal sequences are affected. Incomplete protein fragments may result from interrupted translation which are then released from the cell and are monitored.

Further, the present invention recognizes  
20 alternately spliced forms of the mRNA for pp2c $\alpha$  giving rise to different sizes and/or function in different tissues and assays are designed to recognize the alternately spliced forms in the appropriate tissues.

The identification of alterations in the gene  
25 product in a specific bodily fluid will indicate the source/location of a tumor. For example, with a tumor in the central nervous system, the gene product would be found in the cerebrospinal fluid. Similarly the location of other tumors or other diseases would  
30 determine which bodily fluids to screen and the converse as would be known to those skilled in the art.

The present invention also provides for a kit for detecting PP2C $\alpha$  activity and/or alteration either at the mRNA level or gene product level. The kit includes  
35 molecular probes for mRNA for PP2C $\alpha$  mRNA and detection means for detecting the molecular probe and thereby the



mRNA. Alternatively, or in addition, the kit can contain probes for detecting the PP2C $\alpha$  gene product. The detecting means are in general are antibodies with high specificity for the gene product or agents which  
5 mimic natural proteins which bind to the PP2C $\alpha$  gene product other agents as known in the art may also be used. The antibodies are made as described herein below and in Example 3, which specifically recognize the PP2C $\alpha$  or PP2C $\beta$  gene products (including on the cell  
10 surface) including polymorphisms thereof, and detection means for detecting the binding of the antibody thereby indicating the presence of the gene product and also distinguishing one from the other.

Where appropriate, the kits can also contain  
15 antibodies directed against secondary gene products that are affected by the alteration in function of the PP2C $\alpha$  gene.

The present invention discloses a method of detecting cancer in a patient by detecting altered  
20 levels of PP2C $\beta$  gene activity compared to normal patients in a specimen isolated from a patient.

The present invention also provides for a kit for detecting PP2C $\beta$  activity. The kit includes molecular probes for mRNA for PP2C $\alpha$  polymorphisms thereof and  
25 detection means for detecting the molecular probe and thereby the mRNA or antibodies or other means of identifying alterations in the level of the gene product over normal controls as described herein.

The present invention provides an antibody, either  
30 polyclonal or monoclonal, which specifically binds to a polypeptide/protein encoded by the PP2C $\alpha$  gene as described in Example 3 herein below. The antibodies of the present invention are used in identifying the gene product of PP2C $\alpha$  and PP2C $\beta$ . The present invention  
35 provides monoclonal and polyclonal antibodies raised against recombinantly produced PP2C $\alpha$ , NDDTDSASTD (SEQ

ID No:1), YKNDDTDSTSTDDMW (SEQ ID No:2), recombinantly produced pp2c $\beta$  and PNKDNDGGA (SEQ ID No:3).

The present invention also provides for isolated and purified peptides NDDTDSASTD (SEQ ID No:1),  
5 YKNDDTDSTSTDDMW (SEQ ID No:2) and PNKDNDGGA (SEQ ID No:3). The peptides can be produced recombinantly.

The invention further provides antibodies that will recognize the special structures at the 5'UTR or the RNA-proteins complexes responsible for the  
10 controlled expression of PP2C $\alpha$ . Antibody which recognizes specifically the special RNA structures is also provided.

In general in preparing the antibody, either the entire pp2c $\alpha$  protein or peptide sequences thereof can  
15 be used as an immunogen as well as polymorphisms thereof. Further, anti-idiotypic antibodies can be made against these antibodies. The antibodies may be either monoclonal or polyclonal. Conveniently, the antibodies may be prepared against a synthetic peptide  
20 based on the sequence, or prepared recombinantly by cloning techniques or the natural gene product and/or portions thereof may be isolated and used as the immunogen. Such proteins or peptides can be used to produce antibodies by standard antibody production  
25 technology well known to those skilled in the art as described generally in Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1988.

For producing polyclonal antibodies a host, such  
30 as a rabbit or goat, is immunized with the protein or peptide, generally with an adjuvant and, if necessary, coupled to a carrier; antibodies to the protein are collected from the sera.

For producing monoclonal antibodies, the technique  
35 involves hyperimmunization of an appropriate donor, generally a mouse, with the protein or peptide fragment

and isolation of splenic antibody producing cells. These cells are fused to a cell having immortality, such as a myeloma cell, to provide a fused cell hybrid which has immortality and secretes the required  
5 antibody. The cells are then cultured, in bulk, and the monoclonal antibodies harvested from the culture media for use.

The antibody can be bound to a solid support substrate or conjugated with a detectable moiety or be  
10 both bound and conjugated as is well known in the art. (For a general discussion of conjugation of fluorescent or enzymatic moieties see Johnstone and Thorpe, *Immunochemistry in Practice*, Blackwell Scientific Publications, Oxford, 1982.) The binding of antibodies  
15 to a solid support substrate is also well known in the art. (see for a general discussion Harlow and Lane *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Publications, New York, 1988) The  
20 detectable moieties contemplated with the present invention can include, but are not limited to, fluorescent, metallic, enzymatic and radioactive markers such as biotin, gold, ferritin, alkaline phosphatase,  $\beta$ -galactosidase, peroxidase, urease, fluorescein, rhodamine, tritium,  $^{14}\text{C}$  and iodination.  
25 Additionally, toxins can be coupled to the antibody for targeted delivery.

The present invention also provides for transgenic human PP2C $\alpha$  gene and polymorphic PP2C $\alpha$  gene, animal and cellular (cell lines) models as well as for knockout  
30 PP2C $\alpha$  models. These models are constructed using standard methods known in the art and as set forth in United States Patents 5,387,742, 5,360,735, 5,347,075, 5,298,422, 5,288,846, 5,221,778, 5,175,385, 5,175,384, 5,175,383, 4,736,866 as well as Burke and  
35 Olson, [1991], Capecchi, [1989], Davies et al., [1992], Dickinson et al., [1993], Huxley et al., [1991],

Jakobovits et al., [1993], Lamb et al., [1993],  
Rothstein, [1991], Schedl et al., [1993], Strauss et  
al., [1993]. Further, patent applications WO 94/23049,  
WO 93/14200, WO 94/06908, WO 94/28123 also provide  
5 information.

The present invention provides vectors comprising  
an expression control sequence operatively linked to  
the nucleic acid sequence of the PP2C $\alpha$  gene and  
portions thereof as well as polymorphic sequences  
10 thereof (see Examples herein below). The present  
invention further provides host cells, selected from  
suitable eucaryotic and procaryotic cells, which are  
transformed with these vectors.

The vectors can be introduced into cells or  
15 tissues by any one of a variety of known methods within  
the art. Such methods can be found generally described  
in Sambrook et al., *Molecular Cloning: A Laboratory  
Manual*, Cold Springs Harbor Laboratory, New York  
(1992), in Ausubel et al., *Current Protocols in  
20 Molecular Biology*, John Wiley and Sons, Baltimore,  
Maryland (1989), Chang et al., *Somatic Gene Therapy*,  
CRC Press, Ann Arbor, MI (1995), Vega et al., *Gene  
Targeting*, CRC Press, Ann Arbor, MI (1995) and Gilboa,  
et al (1986) and include, for example, stable or  
25 transient transfection, lipofection, electroporation  
and infection with recombinant viral vectors.  
Introduction of nucleic acids by infection offers  
several advantages over the other listed methods.  
Higher efficiency can be obtained due to their  
30 infectious nature. Moreover, viruses are very  
specialized and typically infect and propagate in  
specific cell types. Thus, their natural specificity  
can be used to target the vectors to specific cell  
types *in vivo* or within a tissue or mixed culture of  
35 cells. Viral vectors can also be modified with  
specific receptors or ligands [Solderling, 1993] to

alter target specificity through receptor mediated events.

More specifically, such vectors are known or can be constructed by those skilled in the art and should contain all expression elements necessary to achieve the desired transcription of the sequences. Other beneficial characteristics can also be contained within the vectors such as mechanisms for recovery of the nucleic acids in a different form. Phagemids are a specific example of such beneficial vectors because they can be used either as plasmids or as bacteriophage vectors. Examples (see Example herein below) of other vectors include viruses such as bacteriophages, baculoviruses and retroviruses, DNA viruses, cosmids, plasmids, liposomes and other recombination vectors. The vectors can also contain elements for use in either procaryotic or eucaryotic host systems. One of ordinary skill in the art will know which host systems are compatible with a particular vector.

Recombinant methods known in the art can also be used to achieve the sense, antisense or triplex inhibition of a target nucleic acid. For example, vectors containing antisense nucleic acids can be employed to express protein or antisense message to reduce the expression of the target nucleic acid and therefore its activity. Additionally, ribozymes can be generated and used to "knock-out" the mRNA expression of the gene [Cech, 1986; Cech, 1990; Hampel et al, 1993; Sullivan, 1994].

A specific example of DNA viral vector for introducing and expressing recombinant sequences is the adenovirus derived vector Adenop53TK. This vector expresses a herpes virus thymidine kinase (TK) gene for either positive or negative selection and an expression cassette for desired recombinant sequences. This vector can be used to infect cells that have an

adenovirus receptor which includes most cancers of epithelial origin as well as others. This vector as well as others that exhibit similar desired functions can be used to treat a mixed population of cells and  
5 can include, for example, an *in vitro* or *ex vivo* culture of cells, a tissue or a human subject.

Additional features can be added to the vector to ensure its safety and/or enhance its therapeutic efficacy. Such features include, for example, markers  
10 that can be used to negatively select against cells infected with the recombinant virus. An example of such a negative selection marker is the TK gene described above that confers sensitivity to the antibiotic gancyclovir. Negative selection is  
15 therefore a means by which infection can be controlled because it provides inducible suicide through the addition of antibiotic. Such protection ensures that if, for example, mutations arise that produce altered forms of the viral vector or recombinant sequence,  
20 cellular transformation will not occur. Features that limit expression to particular cell types can also be included. Such features include, for example, promoter and regulatory elements that are specific for the desired cell type.

25 In addition, recombinant viral vectors are useful for *in vivo* expression of a desired nucleic acid because they offer advantages such as lateral infection and targeting specificity. Lateral infection is inherent in the life cycle of, for example, retrovirus  
30 and is the process by which a single infected cell produces many progeny virions that bud off and infect neighboring cells. The result is that a large area becomes rapidly infected, most of which was not initially infected by the original viral particles.  
35 This is in contrast to vertical-type of infection in which the infectious agent spreads only through

daughter progeny. Viral vectors can also be produced that are unable to spread laterally. This characteristic can be useful if the desired purpose is to introduce a specified gene into only a localized number of targeted cells.

As described above, viruses are very specialized infectious agents that have evolved, in many cases, to elude host defense mechanisms. Typically, viruses infect and propagate in specific cell types. The targeting specificity of viral vectors utilizes its natural specificity to specifically target predetermined cell types and thereby introduce a recombinant gene into the infected cell. The vector to be used in the methods of the invention will depend on desired cell type to be targeted and will be known to those skilled in the art. For example, if breast cancer is to be treated then a vector specific for such epithelial cells would be used. Likewise, if diseases or pathological conditions of the hematopoietic system are to be treated, then a viral vector that is specific for blood cells and their precursors, preferably for the specific type of hematopoietic cell, would be used.

Retroviral vectors can be constructed to function either as infectious particles or to undergo only a single initial round of infection. In the former case, the genome of the virus is modified so that it maintains all the necessary genes, regulatory sequences and packaging signals to synthesize new viral proteins and RNA. Once these molecules are synthesized, the host cell packages the RNA into new viral particles which are capable of undergoing further rounds of infection. The vector's genome is also engineered to encode and express the desired recombinant gene. In the case of non-infectious viral vectors, the vector genome is usually mutated to destroy the viral packaging signal that is required to encapsulate the

RNA into viral particles. Without such a signal, any particles that are formed will not contain a genome and therefore cannot proceed through subsequent rounds of infection. The specific type of vector will depend upon the intended application. The actual vectors are also known and readily available within the art or can be constructed by one skilled in the art using well-known methodology.

The recombinant vector can be administered in several ways and in combination with a suitable pharmaceutical carrier. If viral vectors are used, for example, the procedure can take advantage of their target specificity and consequently, do not have to be administered locally at the diseased site. However, local administration can provide a quicker and more effective treatment, administration can also be performed by, for example, intravenous or subcutaneous injection into the subject. Injection of the viral vectors into a spinal fluid can also be used as a mode of administration, especially in the case of neuro-degenerative diseases. Following injection, the viral vectors will circulate until they recognize host cells with the appropriate target specificity for infection.

An alternate mode of administration of a PP2C $\alpha$  vector can be by direct inoculation locally at the site of the disease or pathological condition or by inoculation into the vascular system supplying the tumor with nutrients. Local administration is advantageous because there is no dilution effect and, therefore, a smaller dose is required to achieve expression in a majority of the targeted cells. Additionally, local inoculation can alleviate the targeting requirement required with other forms of administration since a vector can be used that infects all cells in the inoculated area. If expression is desired in only a specific subset of cells within the



inoculated area, then promoter and regulatory elements that are specific for the desired subset can be used to accomplish this goal. Such non-targeting vectors can be, for example, viral vectors, viral genome, plasmids, phagemids and the like. Transfection vehicles such as liposomes can also be used to introduce the non-viral vectors described above into recipient cells within the inoculated area. Such transfection vehicles are known by one skilled within the art.

In a preferred embodiment, a virus vector based on modified AAV is used. AAV has been shown to integrate into the human genome in chromosome 19q13.3. Alteration of the AAV genome in a mode that will allow it to integrate in a site specific manner into the PP2C $\alpha$  regulatory region is used. (see Example 7)

The invention further provides a method of treating cancer including the steps of first determining the type of cancer and cells expressing the cancer and then preparing a vector as described herein above which will specifically target the cancer cells and includes regulatory elements to control the expressibility of PP2C $\alpha$ . The vector is then administered to the patient and can include a suitable pharmaceutical carrier which will not affect bioactivity of the vector. Alternatively an antisense vector can be prepared and used to control the expression of PP2C $\alpha$ .

pp2c is a protein serine/threonine phosphatase [Cohen 1989]. It is unique among phosphatases since it requires magnesium and is not sensitive to certain phosphatase inhibitors such as okadaic acid [Cohen 1991]. The pp2c family consists of two cytoplasmic isoenzymes in mammalian tissues [McGowan and Cohen, 1987] and at least three pp2c-like enzymes in yeast show the same enzymatic and biochemical properties. The two mammalian isoenzymes are monomers but differ

slightly in molecular mass (44KDa and 42KDa) and are designated pp2c $\alpha$  and pp2c $\beta$ . There is 70% homology between the  $\alpha$  and  $\beta$  isoforms. At the carboxy terminal of the pp2c $\alpha$  there is a fifteen amino acid sequence  
5 which differs from pp2c $\beta$ . In humans the sequence is YKNDDTDSTSTDDMW (SEQ ID No:2).

A 106kb cosmid coding for pp2c $\alpha$  and additional proteins FosB and ERCCI has been sequenced [Martin-Gallardo et al., 1992] (GENBANK accession  
10 number: M89651). Further, the cDNA sequences of PP2C $\alpha$  in humans is known [Mann et al, 1992]. However, attempts to align the 5'UTR of the cDNA with the genomic sequences were not successful.

A hypothesis for the above observations can be  
15 made, but it is not to be construed as limiting the present invention to this one mode. Applicants propose that the UTR consists of several small exons with large introns and propose that PP2C $\alpha$  and FosB have a common regulatory region (ERCCI may also share the regulatory  
20 region). Alternatively, it is possible that PP2C $\alpha$  is a very large gene and that the 5' end and the control region do not reside within the 106kb cosmid in a region located 5' to the 106kb cosmid. As a further alternative, the region of 9kb from the cosmid was not  
25 sequenced due to the high G/C content and it may contain the 5'UTR region and the promoter.

In a preferred embodiment the AAV virus and/or CHINT or other regulatory sequences related to the PP2C $\alpha$  gene are used in the vector, particularly those  
30 which are used to treat patients. CHINT is a cellular sequence which was recombined into the AAV in 9-3 cells; the sequence is set forth in Table 5 (int.li; SEQ ID No:19). The vector can either integrate into the regulatory control of PP2C $\alpha$  and alter its  
35 expression in the same way as AAV alters cells into which it integrates as it is an oncosuppressive virus.

This thereby reinstates normal cellular growth, or there is growth arrest or there can be activation of programmed cell death - apoptosis pathway depending on the cell type, type of cancer, stage of  
5 differentiation, and other factors as known to those skilled in the art. [Schlehofer, 1994]

There are several elements in AAV and/or CHINT or in the PP2C $\alpha$  regulatory elements that can be used to control PP2C $\alpha$  expression taking advantage of the  
10 following observations:

1. PP2C $\alpha$  has a very long 5' and 3' UTR (they are larger than the coding capacity). Specific folding of the RNA and interaction with specific sets of proteins might effect its expression dramatically. At certain stages  
15 there might different modes of folding and these different proteins may interact with the RNA and alter its expression.
2. The CHINT sequences involved in the integration have very interesting motifs which might be used for the  
20 site specific integration. Moreover, applicant has data demonstrating that specific AAV sequences adjacent to the AAV integration site are responsible for the suppression of DNA amplification. These sequences can be used in vectors as a therapeutic and are described  
25 herein below as silencer (SEQ ID No:13) and mini-silencer (SEQ ID No:14).

The invention further provides a method of treating cancer by using an AAV based vector or other vector for cancer treatment that only functions  
30 specifically in cells in which PP2C $\alpha$  is improperly activated. The vector is administered to those who have been diagnosed with a tumor as is known to those skilled in the art. The AAV vector (or other regulatory factor as disclosed herein) in one  
35 embodiment is under the control of a promotor, rep, that is expressed in transformed cells. The integrated

vector will control PP2C $\alpha$  expression in the cell reversing transformation as shown in the Examples. Further, the vector will be targeted to the cell type that has been transformed.

5 Further, since the gene product of PP2C $\alpha$  is expressed on the cell surface, antibody directed against the gene product can activate/inactivate the expression of the gene via the signal transduction pathway. Therefore antibodies can be used  
10 therapeutically to treat patients in which the PP2C $\alpha$  gene needs to be re-regulated. The use of Fab fragments and other means known in the art can be used to insure that the antibodies upon administration to a patient do not have secondary unwanted effects.  
15 Alternatively, a ligand or other molecule which can specifically bind to the PP2C $\alpha$  gene product can be used. The present invention therefore provides a method of binding the gene product of PP2C $\alpha$  expressed on the surface of a cell to induce signal transduction thereby suppressing the transformed phenotype.  
20

The invention further provides a method of treating diseases due to aberrant phosphorylation due to alteration of expression of PP2C $\alpha$  by controlling PP2C $\alpha$  expression. Such disease can be neurologic. For  
25 example, behavioral changes could be associated with aberrant phosphorylation. As shown by Brown et al, 1996 mutations in fosB can lead to behavioral changes. As discussed herein above, fosB and PP2C $\alpha$  are on the 106kD cosmid. There is some indication that they may  
30 be co-regulated. Therefore aberrant expression of PP2C $\alpha$  can be expressed as behavioral changes. Further, the levels of PP2C $\alpha$  activity are extremely high in cardiac and kidney tissues compared to other tissues. Therefore alterations in PP2C $\alpha$  activity will be  
35 reflected in these tissues.

The present invention provides a method of suppressing gene amplification by interrupting the binding or action of DNA polymerase  $\alpha$  primase and RNA polymerase II with the gene product of PP2C $\alpha$  by preparing an antisense vector which will specifically target the binding region of DNA polymerase  $\alpha$  primase and RNA polymerase II to the PP2C $\alpha$  gene product and delivering the vector to the cells as based on the observations set forth in Example 9. Applicants have observed that in tumor cells pp2c $\alpha$  binds to the CTD domain of RNA polymerase II. Therefore alternatively, delivery of a peptide with the CTD domain can be used via competitive binding strategies to control the binding leading to gene amplification.

To investigate the role of AAV in tumor suppression an AAV/neo virus JDT277 was introduced into SV40 transformed Chinese hamster (CO60 and OD4 cells) and mouse mammary tumor cells (DA3). CO60 is a cell line of SV40 transformed Chinese hamster embryo cell lines [Lavi, 1981]. The OD cell line was established by transfection of Chinese hamster embryonic cells with origin deleted SV40 DNA [Lavi, 1985]. The mouse DA3 cell line was derived from mammary tumors syngeneic to BALB/c mice [Sotomayor et al, 1991]. JDT277 virus contains the portion of the AAV2 genome, which encodes the viral Rep proteins, the AAV terminal inverted repeats (TIRs) and the prokaryotic neomycin phosphotransferase gene (neo), conferring resistance to G418. The neo gene was inserted at nucleotide 1882, resulting in carboxy terminus truncated Rep proteins. The truncation of the rep proteins does not affect the ability of the AAV/neo virus to replicate in Adenovirus coinfecting human cells.

Single colonies were isolated and amplified by serial passages in the presence of G418. The resistant cells were designated 9-1 to 9-5 for cells derived from

CO60 cells and A20-A29 for cells derived from OD4 cells. The cell lines derived from the mouse cells DA3 were designated J1-J15.

Alterations of the transformed phenotype:

- 5 Following AAV integration the cells lost several of their transformed characteristics.

1. Suppression of SV40 DNA amplification. (Example 7)

A characteristic trait of tumor cells is their capability to amplify DNA. CO60 cells are used as a  
10 model system to study gene amplification and SV40 amplification can be induced in the cells as a results of treatment with carcinogens [Lavi, 1981; Aladjem and Lavi, 1992]. Following AAV/neo virus integration, the cells were incapable to amplify SV40. Most AAV/neo  
15 cell lines derived from CO60 cells lost their capability to amplify SV40 upon treatment with carcinogen in contrast to the parental CO60 cells [Tal Burstyn, 1993]. Extracts from AAV/neo cells derived from both OD4 and CO60 cells lost their capability to  
20 amplify SV40 in vitro [Winocour et al., 1992; Tal Burstyn, 1993].

2. The cells harboring the integrated SV40 became highly sensitive to treatment with UV or MNNG. [Winocour et al., 1992].

25 3. The transformed cells lost their capability to grow in soft agar, a characteristic typical to transformed cells.

4. The cells displayed apoptotic phenotype as measured by

30 a) the cell cycle pattern was altered and apoptotic cells appeared spontaneously in the AAV/neo cells and the level was enhanced following treatment with DNA damaging agents. (Figure 1A, Table 3A and Figure 1B, Table 3B),

35 b) the condensation and fragmentation of the chromatin and cytoplasm. Condensation and

fragmentation of chromatin was monitored by staining with acridine orange. Ethidium bromide did not stain these cells.

Acridine orange is taken up by both live and dead cells creating fluorescent-green signals, whereas ethidium bromide is only taken up by nonviable cells and gives bright red fluorescence. This double staining system provides a means to distinguish between dead and living cells, and cells that underwent apoptosis before they lost their membrane integrity. Both normal or apoptotic nuclei in living cells fluoresce bright green. In striking contrast, normal and apoptotic nuclei in dead cells fluoresce bright red.

A substantiated fraction of the cells displayed apoptotic nuclei showing condensed chromatin upon staining with acridine orange. In addition, the cells displayed a strong shrinkage of the cytoplasm. Often the nuclei were disrupted into a multitude of micronuclei. These cells underwent apoptosis without losing their membrane integrity. EtBr did not penetrate into these cells, thus the cells were still alive. A large amount of living apoptotic nuclei were found in the treated AAV positive cells compared to a considerably lower percentage in the treated (7.5  $\mu$ g/ml MNNG) and control CO60 cells. The same pattern of staining repeated in all the AAV/neo cell lines. Hence, this apoptotic phenotype was a common feature to all the AAV/neo cells.

c) by the breaks in the chromosomal DNA:

Programmed cell death was shown to be associated with DNA fragmentation. This approach to detect apoptosis was based on the specific binding of terminal deoxynucleotidyl transferase (TdT) to 3'-OH ends of DNA, ensuing a synthesis of polydeoxynucleotide polymer [Gavrieli et al., 1992]. By adding

biotinylated deoxyuridine to fixed cells, TDT was used to incorporate these nucleotides at sites of DNA breaks. The signal was amplified by FITC-Avidin binding enabling identification by fluorescence microscopy. In all AAV/neo cell lines, Applicants could detect a distinct pattern of nuclear staining, directly correlated to the typical degradation of chromatin in apoptotic cells. Since this reaction is specific, only the apoptotic nuclei are stained. As a positive control for the efficiency of the technique, Applicants used CO60 nuclei treated with DNase.

C9-2 and C9-3, the AAV positive clones displayed a bright nuclear fluorescence 48 hours after treatment with 2.5 $\mu$ g/ml MNNG, whereas the controls without any treatment showed only a low background fluorescence. Applicants could see the characteristic degradation of the nuclei into a multitude of small highly fluorescent micronuclei. The fluorescence obtained was similar to the positive control.

Untreated AAV/neo cells and control and treated CO60 (7.5  $\mu$ g/ml MNNG) did not show any sign of fluorescence. This pattern of nuclear degradation appeared in all AAV/neo cell lines tested (approximately 20), however, the extent of fragmentation varied in the different lines.

Unexpectedly, the revertant cells, designated C9-3-2 and C9-3-12, which were selected on the basis of loss of resistance to G418, and lost their integrated AAV sequences [Burstyn, 1993], still maintained their apoptotic phenotype following treatment with 2.5 $\mu$ g/ml or 5 $\mu$ g/ml MNNG.

Further analysis by FACS, Giemsa staining, and electrophoretic separation of the nucleosomal DNA fragments from high molecular weight cellular DNA supported these results.



The integrated AAV in the AAV/neo cells (Examples 6 and 7)

Analysis of cell extract derived from the Chinese hamster A20-A29, 9-1 to 9-5 and the mouse DA3 derivatives for the expression of rep proteins by immunoblotting with anti-Rep antibodies demonstrated that in all cell lines the authentic Rep products were not present. In some cell lines a short protein, probably a truncated protein, reacted with the anti-Rep antibodies [Winocour et. al., 1992].

PCR analysis of most cell lines for the presence of the intact rep promoter region demonstrated that in most cell lines the promoter region of the Rep protein was reorganized as a result of deletions, insertions and rearrangements of the AAV sequences thus eliminating the expression of the authentic Rep proteins.

Applicant focused on the analysis of one Chinese hamster cell lines, 9-3, derived from CO60 cells (Figure 3). The integrated AAV undergoes duplication in this cell line and the chromosome harboring the AAV contains two regions in which AAV is integrated. This duplication of AAV probably resulted from the massive rearrangement which occurred in the Chinese hamster genome following AAV integration. In all Chinese hamster cell lines studied so far by *in situ* hybridization (6 independent cell lines) the chromosome harboring the integrated AAV was altered and was different in many respects from all the typical Chinese hamster chromosomes, thus the identity of the chromosome could not be established.

The integrated AAV and flanking cellular sequences for 9-3 were cloned into a phage (Figure 3). As diagramed in Figure 3 the viral genome underwent several changes. Sequences downstream to the AAV p5 promoter were deleted and replaced by a cellular

fragment "CHINT". In addition, deletions and rearrangements in the 5' portion of the AAV/neo genome were observed. In contrast, the region coding for the Neo gene and the 3' end of the viral genome remained  
5 intact. (Similar alterations were observed in all AAV/neo Chinese hamster and mouse cell lines tested).

The "CHINT" sequences were used as a probe to analyze AAV integration site in different AAV/neo Chinese hamster clones (Figure 2). In several AAV/neo  
10 clones there was a shift in this fragment suggesting that the size of this fragment was altered upon AAV integration. Some of the shifted bands also hybridized to AAV probe demonstrating that AAV indeed integrated into this region. A probe from the subcloned plasmid  
15 pSL9-6 derived from the flanking cellular sequences (Figure 3) was hybridized to the mouse DA3 AAV/neo cells and in most cases it was associated with AAV integration. These results suggest that the AAV integration site in Chinese hamster might be similar to  
20 the one in mouse cells.

Sequence comparison using Genetic Computer Group Inc. software demonstrated that the CHINT sequences are 58.3% homologous to a sequence in the human chromosome 19q13.3. This human sequence is a part of a 106Kb  
25 fragment which was automatically sequenced and analyzed [Martin-Gallardo et al., 1992] (GENBANK accession number: M89651). The region which showed homology to the CHINT sequences was a part of the gene coding for human type protein phosphatase 2C (pp2c $\alpha$ ). According  
30 to the cDNA sequence of this gene the exact region homologous to CHINT is located upstream to the 5'UTR of PP2C $\alpha$  (Table 5) (GENBANK accession numbers: human PP2C $\alpha$  S87759, rabbit PP2C $\alpha$  S87757).

Using a PCR fragment derived by using two primers  
35 (Primers 1 and 4, Methods herein below) for PP2C $\alpha$ , cDNA DNA from 9-3 was probed and a XbaI fragment was found

-31-

which hybridized to AAV, CHINT and PP2C $\alpha$ , and an *EcoRI* fragment was found which hybridized to PP2C $\alpha$  and CHINT in CO60 DNA. Thus, PP2C $\alpha$  is indeed localized in very close proximity to CHINT in CO60 cells and is at the integration site in 9-3 cells. In situ hybridization confirmed this conclusion (Figure 4A).

In both Chinese hamster (CO60 and OD4) and mouse cells (DA3) a portion of the PP2C $\alpha$  sequences was adjacent to the CHINT hamster sequence present in pSL9-6 (Figure 3) which was derived from the lambda clone of the AAV integration site. Thus in both the normal Chinese hamster and mouse chromosome PP2C $\alpha$  is indeed localized at very close proximity, less than 4Kb from the sequences surrounding the integrated AAV (Figure 4B).

Furthermore, in mouse DA3 cells harboring the integrated AAV genome the AAV sequences were associated with PP2C $\alpha$  or with fragment 6 or with both (Figure 5). The integrated AAV was cloned from DA3J7 ( $\lambda$ DA37A) and parts of the phage were sequenced as shown in the figure. Homology was found to a region within the integrated AAV in the  $\lambda$ SL9-1 plasmid (Figure 3) and to the human chromosome 19q13.3 in the position 23467 - 23715 in cosmid MMDA (Access #M63796) which was automatically sequenced and contained PP2C $\alpha$  first coding exon in position 59770 in MMDBC, GenBank accession #M89657, as well as 35-3.seg and 35-T7 as shown in Figure 3. MMDA and MMDBC are two cosmids in the same contig. (More details on the sequences are found in Example 10 herein below).

Little is known about the role of PP2C $\alpha$  in the cell or about its native substrates mainly because of the lack of specific inhibitors for PP2C $\alpha$ . In *Schizosaccharomyces pombe* it has been shown that pp2c $\alpha$ -like enzymes are important for heat shock response and in osmoregulation [Shiozaki, 1995;

Shiozaki, 1994]. In *Saccharomyces cerevisiae* pp2c-like activity has been implicated in the regulation of tRNA splicing and cell separation [Robinson et al., 1994]. In neural cells pp2c $\alpha$  might have a role in the regulation of the Ca<sup>++</sup>-independent activity of Ca<sup>++</sup>/calmodulin dependent protein kinase II [Fukugana, 1993]. Otherwise, information about pp2c $\alpha$  is scarce.

pp2c $\alpha$  might be a cell marker itself. The prior art does not provide information about pp2c $\alpha$  expression in tumor cells. There is a publication suggesting that pp2c $\alpha$  might have a role during myogenic differentiation. [Ohishi, 1992]. Based on the presence of a 10 amino acid motif which appears also in other transcription factors, pp2c $\alpha$  might function like a transcription factor and might regulate transcription in the cell under specific growth conditions and tissues. It can thus behave like E2F which is a major transcription factor and can act when improperly expressed either as an oncogene or as a tumor suppressor factor [Weinberg, 1996]

Unexpectedly, analysis of the PP2C $\alpha$  mRNA in the AAV/neo cells demonstrated that the transcription of the gene was reduced upon treatment with DNA damaging agents in contrast to the parental SV40 transformed cells in which PP2C $\alpha$  was induced following the treatment (Figure 6).

The following is the densitometry analysis of the hybridization signals

	CO60 C	CO60 T	<u>CO60 T</u> CO60 C	C9-3C	C9-3T	<u>C9-3T</u> C9-3C
PP2C $\alpha$ rRNA	0.15	0.31	2.06	0.26	0.15	0.57

The transcription of PP2C $\alpha$  in C9-3 was reduced upon treatment with MNNG in contrast

to the parental SV40 transformed cells in which PP2C $\alpha$  was induced following treatment.

The intact cDNA coding for pp2c $\alpha$  was cloned from a  
5 cDNA library (provided by M. Oren, the Weizmann  
Institute of Science, Rehovot) and PP2C $\alpha$  clone was  
stably introduced into the AAV/neo cells which lost the  
transformed phenotype following AAV-integration. The  
transformed features were rescued following the  
10 expression of the PP2C $\alpha$  clone in the PP2C $\alpha$  neo cells,  
the cells regained the properties of transformed cells,  
grew in soft agar and lost their apoptotic phenotype,  
but the cells could not be propagated. Similar cells  
which were transfected in the same efficiency with a  
15 control plasmid containing a truncated cDNA of non  
relevant gene did not regain the capability to grow in  
soft agar.

From these studies it is apparent that PP2C $\alpha$  has a  
key role in the initiation and/or maintenance of  
20 transformed cells. It should be noted that though the  
cells grew in soft agar applicant was not able to  
isolate viable cells suggesting that unbalanced  
expression of the cells led to aberrant growth and to  
cell death.

25 PP2C $\alpha$  appears to be important in development. The  
high conservation of the gene and protein throughout  
evolution, the specific control signals including IRES  
(internal ribosome entry site) at the 5'UTR support  
this. The findings by other laboratories that AAV  
30 infection effects specifically tumor cells might have  
two explanations: 1) The virus does not infect normal  
cells or cannot integrate into their genome in a  
specific manner. 2) Alternatively, if AAV integrates  
into PP2C $\alpha$  in normal cells the disruption of this gene  
35 might not effect them or might be lethal not allowing  
the survival of such cells. The fact that the

inactivation of only one allele of PP2C $\alpha$  is responsible for the changes in the transformed phenotype and that the introduction of a functional PP2C $\alpha$  cDNA clone rescues the transformed phenotype demonstrates the importance of PP2C $\alpha$ . Applicant also noticed that in highly tumorigenic Chinese hamster cells derived from 9-3 that the whole chromosome carrying PP2C $\alpha$  is duplicated 3, 4, and even 5 times.

In human, on the same chromosome, in close proximity to PP2C $\alpha$  there is an important tumor specific marker called cancer embryonic antigen (CEA), which appears in most tumor cells. Both genes are mapped to chromosome 19q13.3. Targeting the treatment to cells carrying a marker like CEA should help. (It might be possible that CEA by itself is not relevant to cancer but it is associated with the enhanced expression of PP2C $\alpha$  or duplication of this chromosomal region contains both genes).

The above discussion provides a factual basis for the use of PPC2 in cancer detection and therapy. The methods used with and the utility of the present invention can be shown by the following non-limiting examples and accompanying figures.

## EXAMPLES

### METHODS

General Methods in Molecular Biology: Standard molecular biology techniques known in the art and not specifically described were generally followed as in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, New York (1992), and in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1994). Polymerase chain reaction (PCR) was carried out generally as in *PCR Protocols: A Guide To*

*Methods And Applications*, Academic Press, San Diego, CA (1990).

Reactions and manipulations involving other nucleic acid techniques, unless stated otherwise, were performed as generally described in Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, and methodology as set forth in United States patents 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057 and incorporated herein by reference.

Antibody Capture Assay: The steps of the method are: (1) binding of antigen to a solid phase; (2) binding of the antibody to the antigen; and (3) binding of a labeled secondary antibody to the complex. By binding constant amounts of rpp2c $\alpha$  to the solid phase, Applicants have used this technique to detect and quantitate monoclonal antibodies during the rounds of cloning, and to compare polyclonal antibodies from different rabbits and bleedings. The assay has also been used to detect pp2c $\alpha$  in crude extracts of tissues and cell cultures.

Immunoblotting: The steps of the method are: (1) preparation of antigen sample; tissue extracts, cell culture extracts or rpp2c $\alpha$  preparations; (2) resolution of the sample by SDS-PAGE; (3) transfer of the separated proteins to a nitrocellulose membrane; (4) blocking nonspecific sites on the membrane; (5) incubation with poly- or monoclonal antibody; and (6) detection by labeled secondary antibody. Applicants have used immunoblotting for characterization of antibodies described herein above and for detection of pp2c $\alpha$  in cell and tissue extracts. [Harlow and Lane]

Immunoprecipitation: The method steps are: (1) immobilization of monoclonal antibodies to a solid matrix (anti-mouse IgG conjugated agarose); (2) binding of antigen to immobilized antibodies; (3) resolution of

bound proteins on SDS-PAGE; and (4) immunoblotting and detection of antigen by affinity purified rabbit polyclonal antibodies. The method has been used to estimate the amount and the molecular mass of different sized pp2c $\alpha$  and  $\beta$  polypeptides that were discovered.

pp2c $\alpha$  Activity Assay: PP2C $\alpha$  gene product is purified from the mouse cells by general procedure, and its activity is assayed by its ability to dephosphorylate [32P] casein [McGowan and Cohen, 1988].

Oligonucleotide Primers for Reverse PCR: Rat PP2C- $\alpha$  cDNA specific primers were used for reverse transcription and PCR. These primers were obtained from General Biotechnology, Rehovot, and used without further purification. The primers' position is according to the rat kidney nucleotide sequence of PP2C $\alpha$  cDNA reported by Tamura et al., [1989] in the Genbank (accession number: Gb\_ro: Ratpp2c, J04503). The approximate position of each primer on the rat PP2C $\alpha$  cDNA:

Primer #1: 5' - AGGATCAAGTCATAATGGGA - 3' (74-93nt - sense) (SEQ ID No:4)

Primer #4: 5' - GCTGGAGTCTGATTTACAAC - 3' (1454-1473nt -anti sense) (SEQ ID No:5)

Antisense RNA: Artificial antisense RNA complementary to the PP2C $\alpha$  gene is synthesized, and transfected to the mouse cells by the method of Inouye [1988].

Identification Of Unique Changes In Gene Expression By Differential Display: In order to detect changes in the expression of cellular genes mediated by AAV integration, the differential display method is used [Liang and Pardee, 1992; McClelland et al., 1995]. This method is directed toward the identification of differentially expressed genes among approximately 15,000 individual mRNA species in a pair of mammalian cell population such as infected and uninfected cells, and recovering their cDNA and genomic clones. The



strategy of the method consists of the following steps:

- (1) Reverse transcription in fractions using a set of anchored primers,
- (2) amplification of cDNA species from each fraction using a set of arbitrary primers and anchored primers by labeled PCR,
- (3) electrophoretic separation of the resulting fragments on sequencing gel,
- (4) reamplification of fragments that are different in the two situations, cloning and sequencing, and
- (5) confirmation of differential expression by an independent RNA analysis technique.

More specifically, total RNA is isolated from cells as described by Sambrook et al. The RNA is reverse transcribed with an oligo dT primer designed to bind to the 5' boundary of the poly A tail. The cDNA is amplified in a PCR reaction with the oligo dT primer and a second 10-mer arbitrary in sequence. 40 cycles of PCR are done in the presence of [35S]-dATP, in the following conditions: 94°C for 30 seconds, 42°C for 60 seconds and 72°C for 30 seconds. The amplified cDNAs are separated on a 6% sequencing gel, then exposed to X-ray film.

Bands of interest (bands that are differentially displayed) are cut out from the gel, and reamplified with the same primers as used to generate the original PCR product. To confirm differential regulation of individual candidate bands, Northern blot hybridizations is performed. Fragments of interest are cloned using a TA cloning Kit, and sequenced. Genes detected by this method are hybridized to Northern blots from the appropriate cells.

Chromatin Structure In Latently Infected Cells: Higher order chromatin structure may affect the transcription of cellular genes. Judging by their increased sensitivity to digestion with DNaseI or micrococcal nuclease, transcriptionally active chromatin regions are less tightly packed than chromatin containing

transcriptionally inactive genes. Chromatin is partially purified and digested by micrococcal nuclease [Roth et al., 1990]. Purified DNA fragments are digested with a unique restriction enzyme to generate a series of fragments with one end defined by micrococcal nuclease and the other defined by the restriction enzyme. Fragments are separated by agarose gel electrophoresis, transferred to nitrocellulose filters and probed with labeled DNA fragments. Naked DNA is purified and processed similarly. Nucleosome position and nuclease sensitive regions are inferred by comparison of fragments from naked DNA and chromatin.

The methylation state of genes can indicate chromatin changes. Gene specific DNA methylation is measured by the methylation assay [Kafri et al., 1992]. In this method, total cellular DNA is digested with methyl-sensitive enzymes, such as *HpaII* or *HhaI*, and specific fragments of DNA that contain these sites are amplified by flanking oligonucleotide primers. If a specific site is methylated, the amplification will proceed normally. On the other hand, the presence of an unmethylated site will result in digestion of the fragment and the subsequent failure to visualize the amplification product. When properly calibrated, this assay is linear over a wide range of DNA concentrations and can be used to accurately measure the degree of DNA methylation at specific sites.

#### EXAMPLE 1

##### EXAMPLE OF STUDIES WITH AAV

##### A. Infection of mouse cells with AAV:

##### A1. Generation of mouse cells with stably integrated AAV:

DA3 cells were infected with the JDT277 AAV/neo hybrid virus according to Winocour et. al. [1992], with slight modifications. Single colonies were isolated

and amplified by serial passages in the presence of the antibiotic G418. The resistant cell lines were designated DA3J.

5 The DA3 cell line was derived from the *in vivo* D1-DMBA-3 mammary tumor syngeneic to BALB/c mice. The DA3 cell line produces tumors in BALB/c mice with the same growth kinetics and expresses the same tumor associated antigen (Ag) on its surface as the parental tumor. The cells express specific markers for tumor  
10 cells, and cease to express specific Ag typical to normal breast cells [Sotomayor et al., 1991].

To assess the influence of AAV on mouse cells, DA3 cells were infected with the JDT277 AAV/neo hybrid virus [Tratschin, 1985]. JDT277 contains the portion  
15 of the AAV2 genome, which encodes the Rep proteins, the AAV terminal inverted repeats (TIRs) and the prokaryotic neomycin phosphotransferase gene (neo), conferring resistance to G418. The neo gene was inserted at nucleotide 1882, resulting in carboxy  
20 terminus truncated Rep proteins. The truncation of the Rep proteins does not affect the ability of the AAV/neo virus to replicate in Adeonvirus coinfecting human cells.

Single colonies were isolated and amplified by  
25 serial passages in the presence of G418. The resistant cells were designated DA35.

A2. Characterization of the AAV genome in the DA3J cells:

a. Southern analysis

30 Genomic DNA isolated from DA3J1 - DA3J7 clones was digested with different restriction enzymes (*Bgl*III or *Eco*RI), electrophoresed and hybridized to radiolabelled AAV DNA. The hybridization pattern is different in each clone, probably due to rearrangement of the AAV  
35 genome. Indeed it is known that integration of AAV DNA

is frequently accompanied by alterations within the viral sequences [Walz and Schlehofer, 1992].

b. PCR analysis

To find whether the rep promoters and ORF's were  
5 present in the DA3J cell lines, PCR reactions were  
carried on 13 clones (DA3J1 - DA3J13), using different  
oligonucleotide primers complementary to the AAV and  
neo sequences. The results demonstrated that parts of  
the viral sequences in each clone were somehow  
10 interrupted. In all the examined cell lines the AAV  
rep ORF's were not intact, thus, impairing the  
expression of the AAV specific proteins.

In two clones, DA3J3 and DA3J4, two AAV molecules  
integrated into the host genome in a head-to- tail  
15 pattern. This finding is in agreement with earlier  
studies showing that the AAV DNA recombined into the  
Chinese hamster host DNA, at least in some cases, as a  
head-to-tail concatamer of more then one viral genome,  
via the terminal sequences of the viral molecule  
20 [Cheung et al., 1980; Walz and Schlehofer, 1992]. The  
integrated AAV from DA3J7 served as a silencer for SV40  
replication in 293 cells in a similar assay to that  
described in Example 7 herein below.

A3. Expression of AAV genes in the infected DA3J  
25 cells:

Cell extracts from the infected DA3 were prepared  
according to Winocour et al. [1992]. Samples from the  
extracts were electrophoresed on a 12% PAGE, and  
immunoblotted with an anti-Rep antibody. The results  
30 showed that, the two major Rep proteins, Rep78 and  
Rep68, are not expressed in any of the infected cells,  
however one of the small Rep proteins, Rep40, is  
expressed in two clones, DA3J11 and DA3J13. These  
results were expected from the PCR analysis that showed  
35 that in all the examined cell lines the rep ORF's were  
not intact.

## B. Site specific integration

### B1. Comparison of the integration site of AAV in the mouse cells with the AAV integration site in the Chinese hamster cells:

5        Genomic DNA from parental DA3 and DA3J clones (DA3J1 - DA3J7) was digested with *Bgl*III or *Eco*RI. Following electrophoresis the blots were hybridized once with the cellular sequence from the virus/cell junction, isolated from C9-3 (psL9-6), and once with  
10       radiolabelled AAV DNA. In three of the cell lines (DA3J3, DA3J4 and DA3J6) the cellular probe and the AAV probe hybridized to common bands. Using PP2C $\alpha$  probe  
15       applicant found in *Bgl*III digested DNA that both AAV and PP2C $\alpha$  hybridized to the same bands. Thus, both in Chinese hamster and mouse, AAV always integrated into the same site in the vicinity of PP2C $\alpha$ . Note that in all cell lines including the parental DA3 cells, PP2C $\alpha$  and the cellular clone 6 probes hybridized to the same band which is the preintegration site.

### 20       C. Effect of AAV on the cellular phenotype:

      The following cytological properties were compared between DA3 infected and parental cells:

#### a. Plating efficiency

25       As shown in Table 1, the plating efficiency of the DA3J cells was reduced compared to the plating efficiency of the parental DA3 cells, by 11% (DA3J2) to 54% (DA3J3).

#### b. Sensitivity to UV irradiation

30       As shown in Table 2, the DA3J cells show increased sensitivity to UV irradiation compared to the parental DA3 cells. There is a decrease of 5% to 55% in the survival rate of the DA3J cells compared to the DA3 cells.

35       These results are in agreement with other studies, which demonstrated that AAV infected cells (Hela, CO60) show reduced plating efficiency, and enhanced

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sensitivity to UV irradiation, compared to uninfected cells [Walz and Schlehofer, 1992; Winocour et al., 1992].

5 It is interesting to note that, DA3J3 shows the lowest plating efficiency, and the highest sensitivity to UV irradiation. This may be due to the fact that DA3J3 contains two integrated AAV molecules, while DA3J1 and DA3J2, contains only one.

c. FACS analysis

10 FACS analysis was performed on DA3, DA3J1, DA3J2, and DA3J3 as described by Vindelov et. al., [Vindelov et al., 1983]. In this procedure no significant changes were observed between the parental DA3 cells and the DA3J cells.

15

**EXAMPLE 2**

CLONING AND EXPRESSION OF PP2C $\alpha$

Full length of the coding region of PP2C $\alpha$  cDNA was isolated from a rat cDNA library. The cDNA was cloned  
20 into the expression vector pET-17b (pET System, Novagen) between the KpnI and NotI restriction sites. *E. coli* cells (BL21-DE3) transformed by the expression plasmid yielded high levels of recombinant pp2c $\alpha$  (rpp2c $\alpha$ ) as observed by a very prominent ~45kDa band on  
25 SDS-PAGE.

Assay of pp2c $\alpha$  activity: Protein phosphatase activity was found in crude extracts of cultures harboring the recombinant plasmid, as measured by the method of McGowan and Cohen [Methods Enzymol. 159: 416-  
30 429, 1988].

Purification of rpp2c $\alpha$ : Overnight cultures were grown at 30°C in LB medium containing ampicillin. The cells were harvested and disrupted by sonication. The sonicate, cleared by centrifugation, was further  
35 purified by ammonium-sulfate precipitation and anion exchange chromatography on DEAE-sephadex.

**EXAMPLE 3****PRODUCTION AND ANALYSIS OF ANTIBODY**

Polyclonal Antibody preparation in rabbits: Crude cell extracts containing ~250-500  $\mu$ g rpp2c $\alpha$  were separated on 12% preparative SDS-PAGE (200x150x1.5 mm). The rpp2c $\alpha$  band, located by side-strip staining, was excised and stored at -20°C. For injection in rabbits, the band was fragmented by repeated passage through a 18 gauge syringe needle and mixed with an equal volume of Freund's adjuvant. Four ml of emulsion originating from one SDS-PAGE band were used to inject two rabbits. The rabbits, which were pre-bled, were injected subcutaneously at four week intervals. For primary immunization complete Freund's adjuvant was used and all other injections were in incomplete Freund's. The animals were bled every two weeks and the serum was cleared by centrifugation. The antibodies were designated 351 and 343. Unless otherwise designated work described herein used 351.

Monoclonal antibody preparation: Purified rpp2c $\alpha$  was used for monoclonal antibody preparation, by mouse hybridoma production as described herein above. Hybridoma colonies were screened by antibody capture assay (see herein below) and by immunofluorescent cell staining. Positive colonies were subjected to two rounds of cloning and screening by the same methods. Finally, eight (8) positive clones were chosen for further study. Antibodies from these clones were collected as tissue culture supernatants and also as ascitic fluid.

**Antibodies Developed Against pp2c $\alpha$  and pp2c $\beta$** 

(1) A rabbit polyclonal designated 801 raised against the carboxy terminal peptide (10 a.a.). This antibody recognizes pp2c $\alpha$  and not pp2c $\beta$ .

The antibody was raised in rabbits and was affinity purified against the rpp2c $\alpha$ . This antibody was used in most of the histochemical analyses.

A rabbit polyclonal antibody raised against  
5 PNKDNDGGA (SEQ ID No:3), the carboxy terminal of pp2c $\beta$ .

(2) A rabbit polyclonal designated 351 raised against rpp2c $\alpha$  which recognizes epitopes on both  $\alpha$  and  $\beta$ .

(3) Eight independent monoclonal antibodies which  
10 were raised against the rpp2c $\alpha$  and were screened and chosen according to their reaction in ELISA with the rpp2c $\alpha$  and by their capability to recognize pp2c ( $\alpha$  and  $\beta$  or  $\alpha$ ) by immunofluorescence staining of hepatoma cells by Western blotting and immunoprecipitation.

15 Table 4 provides the characterization of eight monoclonal antibodies by antibody capture assay, immunoblotting and immunoprecipitation. The combination of these assays allow the isolation of monoclonal antibodies with the proper specificity of  
20 the present invention.

#### EXAMPLE 4

Expression of pp2c $\alpha$  in normal Breast tissue and Breast tumors: Paraffin blocks obtained from normal breast  
25 and breast carcinoma were stained with 801 antibodies specific to pp2c $\alpha$  and then with secondary antibodies coupled to peroxidase. The substrate was DAB. The samples were counterstained with methylene blue. In a few experiments, the antibodies used were monoclonal  
30 2A3 which are specific to pp2c $\alpha$ . The magnification was  $\times 400$ . Normal liver and hepatoma tissue and normal colon and colon carcinoma tissue was also tested.

In the normal and hyperplastic breast samples, the nuclei were stained very predominantly with the  
35 antibodies. In breast carcinoma, there was a predominant staining in the cytoplasm. In invasive



carcinoma, no staining with anti-pp2c $\alpha$  was observed. Interestingly, in liver tissue cultured cells, when stained with 801 or 2A3 antibodies, the cell surface was stained indicating that PP2C $\alpha$  gene product was  
5 expressing in the cell membrane. In normal liver cells a very pronounced cytoplasmic staining with few very strong nuclear regions stained. In the hepatoma fainter cytoplasmic staining and less staining in the nucleus was observed.

10 It should be noted that it appears there is a differential loss of pp2c $\alpha$  as the cells are advanced in their malignant appearance in the counterstaining.

#### EXAMPLE 5

15 Expression of pp2c $\alpha$  in normal Colorectal tissue and Colorectal tumors: The level of the protein phosphatase 2C $\alpha$  (pp2c $\alpha$ ) expression was assessed in colorectal cancer tissues in comparison to normal colon tissues and in Chinese hamster embryo (CHE) cell line  
20 in comparison to the non permissive SV40 transformed Chinese hamster cells (CO60), by the reverse transcriptase polymerase chain reaction assay (RT-PCR).

Samples of 1  $\mu$ g total RNA were denatured at 65°C for 10 minutes in the presence of 0.5M oligo dT (15  
25 mer) as an anti-sense primer, and immediately chilled on ice. First strand cDNAs were obtained after 60 minutes at 37°C in a 50  $\mu$ l reaction mixture containing: 0.25mM dNTPs (Promega), 10mM DTT, 20u RNasin, 50u MMLV reverse transcriptase and 5  $\mu$ l of 10 x reaction buffer  
30 (STRATAGENE). Following inactivation at 95°C for 10 minutes, 3  $\mu$ l of the resulting cDNA were used in a 100  $\mu$ l PCR reaction containing: 0.025 mM dNTPs (PROMEGA), 10mM DTT, 20U RNasin, 50U MMLV reverse transcriptase and 5  $\mu$ l of 10x reaction buffer (STRATAGENE).

35 Following inactivation at 95°C for 10 minutes, 3  $\mu$ l of the resulting cDNA were used in a 100  $\mu$ l PCR

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reaction containing: 0.025mM dNTPs (PROMEGA), 0.5  $\mu$ M of PP2C $\alpha$  sense primer 5'-GAAGTAGTCGACACCTGT-3' (SEQ ID No:6), 0.5  $\mu$ M of PP2C $\alpha$  anti-sense primer 5'-GCTGGAGTCTGATTTACAAC-3' (SEQ ID No:5), 10 $\times$  reaction buffer, 2.5mM MgCl<sub>2</sub> and 2.5u of ABTaq polymerase (Advanced BioTechnology). Thirty-five cycles of PCR were performed in the following conditions: 1 minute at 94°C, 1 minute at 60°C, 1 minute at 72°C, followed by 72°C for 10 minutes. The same cDNAs were used as templates for parallel PCR reactions performed in the presence of  $\beta$ -actin primers 5'GTTTGAGACCTTCAACACCCC-3' (SEQ ID No:7) and 5'GTGGCCATCTCTTGCTCGAAGTC-3' (SEQ ID No:8), in the same PCR reaction mixtures. Aliquots were taken after 20, 25, 30 and 35 cycles and analyzed by gel electrophoresis.

#### Results

The use of PP2C $\alpha$  specific oligonucleotide primers, generated RT-PCR products with the expected size of 480bp. The RT-PCR reactions demonstrated that in 7 out of 8 samples, the level of PP2C $\alpha$  mRNA were significantly higher in normal colon tissues than the levels obtained in the adjacent tumor colonic tissues. (Figure 7). The level of PP2C $\alpha$  expression in CHE cell line was higher than in the CO60 cell line (Figure 8).

### **EXAMPLE 6**

#### PRODUCTION OF VECTORS AND TRANSFORMED CELLS HARBORING THE VECTOR

Expression of the PP2C $\alpha$  mRNA under the inducible tet promoter: Expression of the sense and antisense PP2C $\alpha$  mRNAs in mammalian cells is based on the system for tetracycline-regulated inducible gene expression as described by Gossen and Bujard [1992].

This system relies on constitutive expression of a tetracycline-controlled transactivator (tTA) fusion

protein which combines the tetracycline repressor with the activating domain of herpes simplex VP16. The tTA was constitutively expressed in rat fibroblasts and in HeLa cells. In these two cell lines the tTA stimulates transcription from a minimal promoter derived from the human cytomegalovirus promoter and the tetracycline operator. Upon addition of tetracycline the stimulation of transcription by tTA is inhibited.

Clones were prepared by stable transfection of the two cell lines with expression vectors that contain the PP2C $\alpha$  mRNA in the sense and in the antisense orientation, under the control of the tTA-dependent promoter.

Construction of expression vectors: The construction of the plasmids used as expression vectors included the following steps:

1. Preparation of the DNA fragment coding for the rat PP2C $\alpha$  mRNA.
2. Cloning of the rat PP2C $\alpha$  cDNA into the tTA containing plasmid.
3. Verification of the plasmids by restriction map analysis.
4. DNA sequence analysis of the PP2C $\alpha$  cDNA insert.

Preparation of the DNA fragment coding for the rat PP2C $\alpha$  mRNA: The DNA fragment coding for the rat PP2C $\alpha$  mRNA was prepared by thermal cycling amplification. The template for the amplification reaction was the insert of plasmid skPP2C (PP2C $\alpha$  cDNA cloned into the sk BLUESCRIPT plasmid).

The upper primer used in the amplification reaction contains the sequence coding for the first six amino acids of the rat PP2C $\alpha$  (Met Gly Ala Phe Leu Asp; SEQ ID No:9). The sequence of the upper primer is the following: 5' CGGGATCCGC ATGGGAGCAT TTTTAGAC 3' (SEQ ID No:10).

The lower primer used in the amplification reaction contains the sequence coding for the last five amino acids and the stop codon of the rat PP2C $\alpha$  (Thr Asp Asp Met Trp \*\*\*; SEQ ID No:11). The sequence of the lower primer is the following: 5' CGCGGATCCT TACCACATAT CATCAGT 3' (SEQ ID No:12).

The ends of the DNA fragments were modified by introduction of *Bam*HI restriction sites at both ends. Cloning of the rat PP2C $\alpha$  cDNA into the tTA containing plasmid: Following amplification of the rat PP2C $\alpha$  cDNA, the DNA fragment was cleaved with restriction enzyme *Bam*HI and cloned into plasmid pUHD10-3 [Gossen and Bujard, 1992] downstream from the tetracycline responsive promoter.

Verification of the plasmids by restriction map analysis: The orientation of the cDNA insert with respect to the promoter was determined by restriction map analysis. Plasmids that contain the cDNA in the sense orientation (pYM001) and in the antisense orientation (pYM002) were selected (Figure 9). DNA sequence analysis of the PP2C $\alpha$  cDNA insert: The sequence of the DNA insert of plasmid pYM001 was determined by automatic DNA sequence analysis. The primers used for sequencing analysis were the same as the one used for cloning. The results of this analysis show that the sequence of the cloned fragment is identical to that of rat PP2C $\alpha$  and that no mutation was introduced during the amplification reaction.

Transfection: Plasmids pYM001 and pYM002 were introduced in the rat fibroblast and in the HeLa cell lines which constitutively express the tTA, by CaPO<sub>4</sub> coprecipitation with plasmid pBSpac. Plasmid pBSpac contains a genetic selective marker, that confers puromycin resistance.

Twenty-four to forty-eight hours following transfection, cells were passaged 1:10 and grown in

selective medium. The selective medium for HeLa and for the rat fibroblast cell lines contained 0.3 ( $\mu\text{g/ml}$ ) and 1  $\mu\text{g/ml}$ , neuromycin respectively. After two to three weeks clones were isolated, grown to confluence in 24 wells culture plates, transferred to 10 cm dish, grown to 70-90% confluence and frozen in 90% fetal calf serum 10% DMSO. In these clones following the removal of Tet we observed induction in PP2C $\alpha$  mRNA in clones harboring pYM001 and reduction in the endogenous mRNA of PP2C $\alpha$  in a clone harboring the antisense plasmid pYM002.

#### EXAMPLE 7

##### Role of integrated AAV

##### in the modulation of PP2C $\alpha$ expression

This work does not identify the precise site of AAV integration within the gene. Additionally, the data does not provide the exact site for the human integration site which is also located on the same chromosomal region 19q13.3 but not within the 106kb cosmid. Applicants hypothesize that based on the results with RNA and the specific protein that the AAV integration site is either in the gene coding for pp2c $\alpha$  or its regulatory region is in some way associated with PP2C $\alpha$ . For example, rep might interact with pp2c $\alpha$  protein and the AAV genome linked to rep and PP2C $\alpha$  might be associated with the regulatory region of PP2C $\alpha$ .

In stable cell lines, SV40 amplification was suppressed by infection with recombinant AAV/neo virus and Applicants were not able to detect the expression of rep or the sequences coding for rep and, therefore, Applicants searched for a sequence with suppressing activity. This sequence was present in all the AAV harboring cells whether they are of Chinese hamster or mouse origin.

A functional correlation between the integrated AAV sequences and the modulation of PP2C $\alpha$  activity in the cells harboring the AAV genome was not demonstrated yet, however, it is clear that as a result of the AAV integration, there was an alteration in the transformed characteristics as described herein.

Based on the studies described below, it seems feasible that in addition to the site specific integration which occurs in the vicinity of PP2C $\alpha$ , the AAV sequences can have some importance in the alteration of the transformed phenotype.

The integrated AAV in SV40 transformed Chinese hamster cells (line 9-3 and other cell lines) is responsible for the suppression of the carcinogen induced SV40 amplification. The viral element responsible for the suppression of SV40 amplification (silencer, SEQ ID No:13) was defined using a transient assay for SV40 replication [Yang et al, 1995] to demonstrate that the AAV rep protein is responsible for the suppression of SV40 replication. This assay is based on transfection of the human kidney cell line 293 with an SV40 vector containing the coding region for T antigen and the viral origin of replication and cotransfection with several different constructs derived from the vicinity of AAV integration in 9-3, SV40 transformed Chinese hamster embryo cells containing an integrated AAV and DA357 (a mouse cell line harboring the integrated AAV in which the transformed phenotype was altered following AAV integration).

Using different constructs, Applicants succeeded to define the minimal AAV element conferring the suppression. This element is comprised of 64 nucleotides from the AAV genome (nucleotides 125-189).

ACTCCATCACTAGGGGTTCTGGAGGGGTG  
GAGTCGTGACGTGAATTACGTCATAGGGTTAGGG

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This element was termed SV40 silencer (SEQ ID No:13) though in an alternative embodiment only 21 nucleotides, 125-145, (SEQ ID No:14) are responsible.

The replication of the SV40 vector in 293 cells results in DNA which is not methylated and, therefore, is cleaved by *DpnII*, an enzyme that cleaves only unmethylated DNA. The *DpnII* digested DNA was separated on a gel, blotted and hybridized with SV40 probe. The results are shown in Figure 12.

The blot provides the following:

Lane 1: Cotransfection with pSK1 and pAV2 (a plasmid containing the whole AAV genome and expressing the rep protein). Note that SV40 replication is suppressed.

Lane 2: Transfection with the SV40 plasmid pSK1 SV40 replicates. Replication of the SV40 template is observed.

Lane 3: Cotransfection of pSVK1 and a plasmid harboring 138bp from the AAV genome (nucleotides 125-263). There was a suppression of SV40 replication by this element.

Lane 4: Cotransfection of pSVK1 with pSL9-6 (non AAV DNA sequences).

Thus SV40 replication in 293 cells was suppressed by rep and by the silencer element.

Similarly SV40 replication was suppressed when the cells were transfected by a plasmid containing only 125-145 (the SV40 mini-silencer, SEQ ID No:14).

5' A<sub>124</sub>C<sub>125</sub>TCCCATCACTAGGGGTTTCCT<sub>145</sub>.

In control experiments in which Applicants transfected with other sequences derived from the integrated AAV and cellular sequences spanning the integrated AAV, such as pSL9-6 and others (see Figure 12, lane 4) no repression of SV40 DNA replication was detected.

Using a  $\lambda$  clone containing the integrated AAV and the flanking cellular sequences from the mouse DA3J7 cell line similar suppression of SV40 replication was

observed. This clone contains the 64 nucleotides comprising the silencer region.

Note that suppression of SV40 replication can be obtained in 293 cells by Rep expression and by the 64bp  
5 silencer element in a transient assay.

Revertants which have lost the integrated AAV regained the capability to amplify SV40. In one revertant line, C9-3-2, applicant showed that the revertants lost the whole chromosome containing the  
10 integrated AAV. Applicants showed this by FISH, the disappearance of a very well characteristic abnormal chromosome into which AAV integrated.

A hypothesis for the above observations can be made, but it is not to be construed as limiting the  
15 present invention to this one mode of action. Applicants propose that the Rep protein [Heilbronn, Schlehofer et al, 1983; Kleinschmidt et al, 1995; Yang et al, 1995] and the silencer element can suppress SV40 replication by interaction with a similar protein or  
20 element directly or indirectly, possibly PP2C $\alpha$

It is possible that the 21 bp (mini-silencer) from AAV genome modulates PP2C $\alpha$  activity as well by interaction and activation of a control region. Alternatively, the silencer can act as a dominant  
25 negative element interacting directly and/or indirectly with proteins associated with the replication of SV40. PP2C can regulate the action of such proteins by dephosphorylation. An example for such interplay can be the DNA polymerase  $\alpha$  primase. It is possible that  
30 the rep protein is directly also involved in such interactions.

It appears that dephosphorylate DNA polymerase  $\alpha$  primase is responsible for the initiation of SV40 DNA replication during the carcinogen induced amplification  
35 in CO60 cells. Moreover that this phosphorylate - dephosphorylate process are controlled by the cell



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cycle. Thus PP2C $\alpha$  can modulate the activity of the DNA polymerase  $\alpha$  primase depletion of PP2C $\alpha$  due to its binding to rep directly or indirectly might lead to aberrant phosphorylation of the DNA polymerase  $\alpha$  primase and to its failure to initiate SV40 DNA replication.

#### EXAMPLE 8

##### THERE ARE MORE pp2c $\alpha$ PROTEINS THAN THE 42kd

Liver extracts were immunoprecipitated with the different monoclonal antibodies raised against rpp2c $\alpha$  (see Example 3 herein above). The precipitates were divided into two aliquots which were separated on 12% PAGE and blotted. Each set of immunoprecipitates was challenged with the following polyclonal antibodies:

- (1) 351 - which recognizes both  $\alpha$  and  $\beta$
- (2) 801 - specific to pp2c $\alpha$

As demonstrated in Figure 10 upon reaction with antibody 351 several bands migrating in the position of 40-43kd were detected. Monoclonal antibodies 9F11, 9F4 and 1D5 displayed a very similar picture of 2-3 strong bands and 1-2 faint bands. Monoclonal antibody 2A3 precipitated only the faint bands.

The second part of the blot was reacted with the  $\alpha$  specific antibody 801. In the position of ~40-43kd, two bands were visible with all four monoclonal antibodies. These bands are probably those which were detected by monoclonal 2A3. Thus, monoclonal antibody 2A3 is specific for pp2c $\alpha$ .

In addition, two additional bands migrating in the position of 75kb and greater than 150kd were detected. These proteins are more abundant in liver than the 40-43kd protein. Since all eight monoclonal antibodies recognized these proteins and since the 801 polyclonal antibody reacted as well, it is clear that these two large proteins share several epitopes with pp2c $\alpha$

suggesting that they are the product of the same gene but result from alternative splicing.

A weak reaction with the 75kd protein is detected with polyclonal antibody 351 when reacted with the immunoprecipitate. However, upon direct immunoblotting of total liver extract, it seems that 75kd was present and a faint reaction could be detected against the higher molecular weight protein.

Several additional higher molecular weight proteins were also detected with polyclonal antibody 351. These proteins did not interact with polyclonal antibody 801, which is specific to pp2c $\alpha$ . These results suggest that other forms of  $\alpha$  exist and have different molecular weights.

Norther blot analysis (Figure 13) of mRNA derived from several tissues displayed several bands of RNA which hybridized with probes derived from the 5'UTR of pp2c $\alpha$  or with the entire PP2C $\alpha$  cDNA probe. The RNA was extracted from different tissues and different sizes of RNA appeared in these tissues.

#### EXAMPLE 9

##### pp2c $\alpha$ REGULATES mRNA SYNTHESIS

Table 6 summarizes the protein or RNA sequence homology that was found for the 10 amino acid pp2c $\alpha$  carboxy terminal peptide: NDDTDSASTD (SEQ ID No:1). This peptide was used to raise polyclonal antibody 801 as described herein above.

The carboxy cellular domain (CTD) of the RNA polymerase II fused to GST and bound the fusion complex to sepharose glutathion beads and mixed with HeLa cell extract. Following PAGE and blot the proteins bound to the carboxy terminal domain (CTD) of the RNA polymerase bound also to pp2c $\alpha$ . Both 801 and 2A3 were used with the blot. The size of the associated pp2c $\alpha$  was

-55-

approximately 43kD. Thus, RNA polymerase II is associated with pp2c $\alpha$ .

In a second experiment, extracts from tumor cells, Hepatoma and HeLA, were assayed as above. Binding was observed to GST-CTD only in the tumor extracts while in cell extracts from normal hepatocytes such activity was not detected.

Based on the studies [Chambers and Dahmus, 1994] which demonstrated that the polymerase  $\alpha$  CTD domain can be dephosphorylated by a phosphatase similar in its properties to PP2C $\alpha$  (but not PP2C $\alpha$ ) applicant proposes that pp2c $\alpha$  dephosphorylates RNA polymerase II and thus regulates the initiation of mRNA synthesis on specific messenger. This peptide can be used to control and regulate transcription facilitated by other factors.

#### EXAMPLE 10

##### FURTHER SEQUENCES

Two  $\lambda$  clones containing the AAV integration site were prepared. (1) One was derived from the chinese hamster cell CO60 designated  $\lambda$ SL9-1 (schematic diagram in Figure 3; SEQ ID Nos:15 and 16). Parts were sequences as indicated in Figure 3. A further sequence AN8T7 (SEQ ID No:18) was derived from plasmid pSL9-8 (Figure 3). (2) The second  $\lambda$  phage was cloned from the cell line DA3J7 and was not mapped. Portions were subcloned to plasmids and part sequenced as set forth in 5h-1 (SEQ ID No:17) A sequence comparison shows that 5h-1 is homologous to AN8T7. This region of comparison was also homologous to the cosmid MMDA 23,467-23715. Throughout this application, various publications are referenced by citation and patents by patent number. Full citations for the publications are listed below. The disclosures of these publications in their entireties are hereby incorporated by reference

into this application in order to more fully describe the state of the art to which this invention pertains.

The invention has been described in an illustrative manner, and it is to be understood that the terminology which has been used is intended to be in the nature of words of description rather than of limitation.

Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

**Table 1.** Plating efficiency of the DA<sub>3</sub>J clones compared with that of the parental DA<sub>3</sub> cell line

No. of cells plated	No. of outgrowing colonies (mean $\pm$ SD), % of plating efficiency			
	DA <sub>3</sub>	DA <sub>3</sub> J <sub>1</sub>	DA <sub>3</sub> J <sub>2</sub>	DA <sub>3</sub> J <sub>3</sub>
250	88 (6) 35%	53 (8) 21%	78 (11) 31%	43 (5) 17%
500	181 (36) 36%	103 (10) 20%	131 (20) 26%	82 (9) 17%

Semiconfluent cells derived from DA<sub>3</sub>, DA<sub>3</sub>J<sub>1</sub>, DA<sub>3</sub>J<sub>2</sub>, and DA<sub>3</sub>J<sub>3</sub> cell cultures were seeded at 250 and 500 cells onto 9-cm plastic petri dishes. Cultures were grown for 7 days. Cells were then fixed and stained with Gimsa. The mean number of growing colonies from two experiments was determined. Each experiment was performed in triplicates.

**Table 2.** UV sensitivity of the DA<sub>3</sub>J clones compared with that of the parental DA<sub>3</sub> cell line

J/m <sup>2</sup>	No. of outgrowing colonies (mean $\pm$ SD), % of survival							
	DA <sub>3</sub>		DA <sub>3</sub> J <sub>1</sub>		DA <sub>3</sub> J <sub>2</sub>		DA <sub>3</sub> J <sub>3</sub>	
0	91 (4)	100%	59 (20)	100%	79 (6)	100%	36 (7)	100%
2.5	86 (9)	95%	54 (9)	91%	85 (8)	100%	36 (4)	100%
5	62 (5)	68%	25 (30)	42%	39 (13)	49%	11 (11)	30%
10	22 (6)	24%	12 (2)	20%	19 (4)	24%	4 (2)	11%
20	0 (0)	0%	0 (0)	0%	2 (2)	2%	0 (0)	0%

Semiconfluently growing cells from DA<sub>3</sub>, DA<sub>3</sub>J<sub>1</sub>, DA<sub>3</sub>J<sub>2</sub>, and DA<sub>3</sub>J<sub>3</sub> cell cultures were seeded at 250 cells onto 9-cm plastic petri dishes. After 48 hours the cultures were irradiated with UV light, and then incubated for 7 days. The mean number of growing colonies was determined. The experiment was performed in triplicates.

Table 3A

File: Shu..008 Acquisition Date: 10-May-95  
Total Events: 7000 X Parameter: FL2-A (Linear)

Marker	Events	% Total	Mean	CV
All	7000	100.00	384.11	48.19
G0/G1	3355	47.93	394.88	14.49
S	943	13.47	569.28	7.41
G2+M	668	9.54	727.76	8.06
Ap.	1485	21.21	132.36	36.21

TABLE 3B

File: Shu..010 Acquisition Date: 10-May-95  
Total Events: 7000 X Parameter: FL2-A (Linear)

Marker	Events	% Total	Mean	CV
All	7000	100.00	382.46	51.58
G0/G1	3019	43.13	393.54	14.79
S	938	13.40	574.35	7.10
G2+M	798	11.40	737.43	8.24
Ap.	1684	24.06	133.69	36.68

Table 4:  
Characterization of Monoclonal Antibodies

Antibody number	Antibody capture (1)		Immuno-blotting (2)	Immuno- (3) precipitation
	PP2C	PET		
1D5	+ 1/8	-	+++	+++
2A3	+ 1/32	-	+++	$\alpha$ -specific
2H8	+ 1/16	+ 1/8	not tested	+++
9F4	+ 1/10	-	+++	+++
9F11/169	-	-	nonspecific	+++
9F11/53	+ 1/10	-	+++	+++
10C6	+ 1/10	+ 1/10	++	+++
10F8	+ 1/1	-	+	+++

(1) Antibody capture assay was performed on two antigens: purified rpp2c - to identify anti pp2c antibodies, and a protein extract from cells containing the pET expression vector without the pp2c insert - to identify non-specific antibodies. Results are expressed as + or - and the number indicates the antibody dilution giving the highest result.

(2) Immunoblotting tested on rpp2c and on liver extracts separated on 12% SDS-PAGE.

(3) Immunoprecipitation. Monoclonal antibodies were used to precipitate proteins from a liver extract. The proteins were separated on 12% SDS-PAGE and detected by affinity purified rabbit polyclonal antibodies - 801 which is  $\alpha$ -specific and 351 which identifies pp2c $\alpha$  and pp2c $\beta$ .

TABLE 5

Homology between CHINT (SEQ ID No:19) and  
a sequence from human DNA from cosmid DNA  
MMDB (SEQ ID No:20) containing the 5' end  
of the PP2C RNA

LOCUS HUMMMDBC 68505 bp ds-DNA PRI 09-APR-1992  
DEFINITION Human DNA from cosmid DNA MMDB (f10080) and MMDC (f13544) from  
chromosome 19q13.3 (obtained by automated sequence analysis).  
ACCESSION M89651 M77823 M77824 M77825  
KEYWORDS  
SOURCE Homo sapiens (library: Lawrist5 vector library of A.V. Carrano)

SCORES Init1: 61 Initn: 150 Opt: 82  
58.3% identity in 103 bp overlap

```

      20      30      40      50      60      70
int.li TAGTGCCGGTCAAGGAAGTGAACGTGCGATTCCGGGACAGGCTACCCACTCCGATCCCAG
      |||
hummdb CCTCACCTCCGCCCTGTTTCGTCCAGGTCCTCCGGGTGAGGCTACCCCGTCGCCGCCA-
      57710      57720      57730      57740      57750      57760

      80      90     100     110     120     130
int.li GAGAAGTTGTCATGGTGAGGGCCACCCTAGGTCTCTGCCCCTGCTGTGTCCCCCATCTTA
      |||
hummdb GAG-CGCGGGGAGGGGAGAGCTTCCTTTGTCTCCTATGCCTCCT---CCCCCATCCCG
      57770      57780      57790      57800      57810      57820

     140     150     160     170     180     190
int.li CCCATCCAGTAGGATCTAGAGGCTGTGCCCCCTTGTGGAATGCACAGAAGTCACAAGCG
      |||
hummdb GCTCTCCTGCGGGCAAGCGCCGAGGGGACACCGGGAGTACCCACCTGAACCTCTGGGG
      57830      57840      57850      57860      57870      57880

```



TABLE 6

Proteins or RNA Sequences Homologous to NDDTDSASTD

Peptide sequence

- #1
- 1) To the different PP2C $\alpha$  protein and mRNAs.
  - 2) To rattus norvegian neurona. pentroxin precursor mRNA.
  - 3) To Xenopus transcription factor IIIA.
  - 4) To Human DNA/RNA binding protein mRNA.
  - 5) To Human transcription factor IIIA.

and to other additional proteins to a lesser extent.

Homologues on the RNA level

- #2
- 1) PP2C $\alpha$  mRNAs
  - 2) Human mRNA homologue to Xenopus transcription factor IIIA.
  - 3) Human DNA/RNA binding protein mRNA.
  - 4) Human transcription factor IIIA.
- #3
- 1) Different forms of PP2C $\alpha$
  - 2) C. elegans cosmid coding for DNA directed RNA polymerase sigma chain.
  - 3) Potato mRNA for pyruvate kinase.
  - 4) C. elegans cosmid.
  - 5) Ictanurid herpes virus  
DNA polymerase helicase
  - 6) A. thabana mRNA for UIsnRNA specific protein UIA.
  - 7) Ascaris lumbricoides small nuclear RNA (snRNA UI-1 UI-2 UI-3 U-I genes).

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Lavi, Sara
- (ii) TITLE OF INVENTION: MANIPULATION AND DETECTION OF PROTEIN  
PHOSPHATASE 2C -PP2CALPHA- EXPRESSION IN TUMOR CELLS FOR  
CANCER THERAPY, PREVENTION AND DETECTION
- (iii) NUMBER OF SEQUENCES: 20
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Kohn & Associates
  - (B) STREET: 30500 Northwestern Hwy.
  - (C) CITY: Farmington Hills
  - (D) STATE: Michigan
  - (E) COUNTRY: US
  - (F) ZIP: 48334
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (vii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Kohn, Kenneth I.
  - (B) REGISTRATION NUMBER: 30,955
  - (C) REFERENCE/DOCKET NUMBER: 2290.00037
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: (810) 539-5050
  - (B) TELEFAX: (810) 539-5055

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 10 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:  

Asn	Asp	Asp	Thr	Asp	Ser	Ala	Ser	Thr	Asp
1					5				10

## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 15 amino acids

-71-

- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Tyr Lys Asn Asp Asp Thr Asp Ser Thr Ser Thr Asp Asp Met Trp  
1                      5                      10                      15

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 9 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Pro Asn Lys Asp Asn Asp Gly Gly Ala  
1                      5

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AGGATCAAGT CATAATGGGA

20

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "Primer"

(iv) ANTI-SENSE: YES

-72-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:  
GCTGGAGTCT GATTACAAC 20

(2) INFORMATION FOR SEQ ID NO:6:  
(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 18 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: other nucleic acid  
    (A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:  
GAAGTAGTCG ACACCTGT 18

(2) INFORMATION FOR SEQ ID NO:7:  
(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 21 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: other nucleic acid  
    (A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:  
GTTTGAGACC TTCAACACC C 21

(2) INFORMATION FOR SEQ ID NO:8:  
(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 23 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: other nucleic acid  
    (A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:  
GTGGCCATCT CTTGCTCGAA GTC 23

(2) INFORMATION FOR SEQ ID NO:9:  
(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 6 amino acids  
    (B) TYPE: amino acid

-73-

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Met Gly Ala Phe Leu Asp  
1 5

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 28 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CGGGATCCGC ATGGGAGCAT TTTTAGAC

28

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 5 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Thr Asp Asp Met Trp  
1 5

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 27 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CGCGGATCCT TACCACATAT CATCAGT

27

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## (2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 64 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid  
 (A) DESCRIPTION: /desc = "Silencer Region"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

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 AGGG 64

## (2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 22 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid  
 (A) DESCRIPTION: /desc = "Mini-silencer region"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

ACTCCCATCA CTAGGGGTTC CT 22

## (2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1573 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid  
 (A) DESCRIPTION: /desc = "35-3.seg (Figure 3)"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

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 ATTATGACAT TAGCTTTGCG TAGTGAAGGC ACAAGCTGCT AAGTGGTTAG GGATGTATTT 120  
 TGCCGTAGCC TGTATCACNC CAGGTCCTGG GCTCGGTTCC TAGCATTACA GGAAAAAGCA 180  
 GGCGGTGGTT GACCTTTAAT GAATGGATTT TTCAATTTAG AAGTTGGTTT CATTTTAAAG 240  
 AATTCAAAAA TGTTCCCAT AGCACTTTGT TTTGACATTG AGATCAGCTG CTAATTGAGG 300  
 TCCAGTATAT ACTTAGAAAA CTGAGCGAAA CTTTGATGGA CACACACACA CACCCCTGTT 360  
 GTTCATTTAA TAATTGAACT AAATAAAATA CTGTTTAGTC ATCCACGTAA GCAAGAGGCC 420

TGTGTAAACA GTATTTGTAT TAGTAAAAAC TTTATAACAT AGTTACATAA TCAGCATCAT	480
TTTTTTTATG GACCTTATAG TTGGCTACTT CACTGGGTTT GTTATAATTT AATCAGACTC	540
CTAAATAGGT TAAATTTCTG AATTGCCTAC TTCAGTTTTG AAGAATTATT TTGTTTCATA	600
ATTTCCCATG CATATCTGGT AAATAATTCT GGATTGTTTC TAAAGGGGAG AGCAAGGTCT	660
CTTATGCAAA GTGAAAATCT AGATATGCTG TTTGTAAGAA TATAATAGTG ATAAAGTAGT	720
GTCCTTTTGC TCAGTGCCTC CATTCTTACC AGGCTGTGAC TGATCTTCAG TATTATTCAG	780
ACAGTCACTA TTAATATATC CGTTGCACAG TGGGGAAATT GAGGGAAGTT AGATAGGCAT	840
CGGGTATCTT AATCATAACT CACATATACC CAGCTGGCTA GTCAGCCTAG CTAAGACAGT	900
TCACACCCAG TTGAGGCAGC TTGCTGTTGG CCATTAGTAG GTAACCTAAT GGCTTGTTTT	960
CTTCACTGGT AAGGTGGGGA TATAATAATG CCAATAATTG CATAATGATT AAAGACATTA	1020
ATATATTCCA TAAAATTTCC TGAATAGTGC TTAGCTGGTA CCCCTCCCCA CACATGCACC	1080
CCAGTCCAAT GTTCAGATGT TTAATTTGTT AAGCCCAGTT AATCCATTCC CCCTAATATC	1140
TTCTCCCAGT TTGAAGAANG TTGAAGAATG TTGGGCTTGT TAGTTTAATT TTTTAAGAAG	1200
CATATCATGT TGCTTTTTTA AAACATGTTT CTTTGGGTTT TGGCTTCCCC TTTTGGAAAG	1260
AATTCCAATT TACACTTATG GAAGAAAGCC ATTGTCCCCT CCAATTTCCC CCCCTGTCCC	1320
TTTCCAATAC AGCCCAACTC CCCATGTTTT GACTTCCTCC CCTGAACCAC CCCGTTCTCC	1380
TGTTTTTCCC TCCCCANAA AAAAAACCCA ATAATTTGAC TTTGGTAATT GAATTTCCCG	1440
CCNGTTAGGC NCCTGAATTG CCGAAATAAT TCCCCGTGC NCCCNGGANT TTTGGCACCC	1500
CCTGCCCCTT AACCTGTTCT GCTGCCCCC ATTTTAAAT GGCTTGCCGC NTTACNCAA	1560
ANACTGCCTT TCC	1573

## (2) INFORMATION FOR SEQ ID NO:16:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2580 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "35-T7.seg (Figure 3)"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CTCGATCTCA CAAAGTCACA GAGCTCTTCG TTTCCCATGA CATCCAGAT ACCATCACAT	60
GCAAGAATAA TGAAGTATC GTCCTCTTCA GACCTTTCAA TATCATGGAC TTCTGGCTCT	120
GGTGAGACGA GCTGCTCTGT GGGACCTTTT CCATGGACAC ATTTGTAATC GAAATCCCCA	180
AGGGCCCTTG ACACAGCCAG AGAGCCATTT ACACGCTGAA TCATCACAGA GCCCCCTGCA	240
TTCTGAATTC GTTCTTTTTT CAGCGGGTTA CTTGGTTTGT GGTCTTGTGT GAAGAAGTGA	300

ACTTTCCTGT TTCTACAAAG CAAACCTCTC GAGTCTCCAC AGTTAATGAA GTAAGTATGT	360
TTGGGGAGAA ATTAAGACCC CCACAGCTGT TTGACCCACT TCCTATCTGC ACCATGTTTT	420
CCTTCCTCCT GACATGACTC CTCATGTTGT TTCCATCAAT CTCCCAGAAA AACCTGTTCC	480
TGATCCCCAT TCCTTTACAT TTTCCACAG AAAGGTGCTC CCTGCAGAGC CTTTTAAAT	540
CCCTGGTTTA TTGGTGATGT TGATTCTNAA CAAATGCTCC ACAGCCAGTA TTTNGGCAAC	600
CTTGAAAAAC CAGCATGCCC ATCCATATAC AGCCAAGAAT GACCATGTTT TCCAGTTCCA	660
CTTTNGGCAA ACCCAATCCA CAGCCGTTNT GCGCATCCTC CCATTTCAAC TCCGCCAAC	720
CNTTGCNTGC TGCNTTAAGC CATATCGCAA CCCATCCCCC CTGCCCCCTG GGGCATTATG	780
CNTTTCATC TTTGGTTGTC TAAAATGCTC CCATTATGAC TTGATCCTCT AGGTCTGCAA	840
AGGAAGAGAA ATAAGAAAGT TAGTAACTGT CTTTGAAACA AAGCACACAT CCAACAGTCT	900
TTTTGAAGCA CCTACGAGAT ACAAGGAAAC GTAAAACTC ATAGGCTATA GCCATAAGCA	960
TTGTTCTACT GACTTGAAA ATGTAGAGAT TAATAAGAAA GGGAAAGGCT GATCAAGTAC	1020
AGCTCAACCA GACAAGCAGC AGATGGAAC AAGTCACCAG GTAAAAGAGA GCTTGTGTC	1080
CTCTCTGTGA TACCAAGGAG GCCCAGCAGT GACCATTAAC TTACATGAAC TAGGCAAGAT	1140
TTCAGGGTGC ATTCATCATA TGTAACCTCT CAATTAAGTT GTGTGTTGAT TAAAAAAAT	1200
AATTCATAGA AACATACAAG TATCTACTAC TTCAGGGAAC CTTAGCTAAG TACTCAGGAA	1260
TGTTGAGAGT TTGATTCCAT GCTATTTAGT TTTGTTTCTA CAACTAGATA CCTTTGGTAA	1320
AAATAAAAAG TAATTACTCA CACTGGTCCA AATTTTCAGT GCCTTGTGCA GGTCAATTCTC	1380
TTAGCTGGA ATTCCCTGCC TCACCTCTTT ACCAACAGAA AAAAAATACA CCTGTTTCTA	1440
TCCTTTGAAA TCCAGTTCAA TTGTTCCCCC TTCCTCCAGA CTTTACAGTC CTTGAAAAAA	1500
ACAAGTTATT AACTACAGAA GTCAGCTTCC ATTTCCAGTT NGGAATGTTT TTTAATGAAC	1560
AATTTTATTG TTCNAAATCT NACNATATGA TAACTAANCN AATGGTAATA ATATTTTCAN	1620
CCCTGCCCTA TGGCCGCTNT TTTTAATCCT NAAAAAATC NAAGGTCTAT TCCNCCCNC	1680
CTTGCCAATA CTTNACANCN CCAGTTCCT GATCTGGAAT GGACCCACAA AGGTCAAGAC	1740
TTAGGTTANC CTTGCTCAC AACTAAAGA AAATCTTAA GGAGAACAGA ATACTGAAGA	1800
GAGAAATGAG GGTGAAGGAC AGTGTTGAG TGACGTTCTG AAACCAGGGG ACTAAANATA	1860
CCANAANTGG TGTTCAGAC AGAAATGGTA TGGAAACTC CTTAGGAAAG AAATGACANN	1920
TNTTGTTCG CAGCAACCC CNACATGGC TTTCTCTTT TCCTTCTGCT GATTAAGTGA	1980
TGCACNTGGT ANAAAAGTCA ACANACCCCT CCTCCACNCA GACTCCACC GAGTACANNG	2040
GCCCATGTGC TCANTACACT CTGCCCCAAA CTCNNANNAT TCATTCNNCT CCCNTGTNA	2100
TTTATNAGGG CCTTCCCNT CAGTTNTCTN ATCNCAACG GANATTANCC TTCCANNAT	2160
TTACCCCN TTTGTACANC ACATNNTGGC NNGTGCCACN GTTANGCGTC GGCNTCCCTG	2220
TTNCACTNCA TCCCTCATCN TTAGGCCANG TTTGATTCTC CNGTGCANAN TTTCCGCANN	2280



ANCNTACCCC TTGCACCNTC CATNTCTNNG GAANAACCTC CGGTTCTGAA TCTNCCCCNN	2340
TCCCGTCNCT CCCCCNTTCT TTCTTTTCTC TANTTTTTTC CNNGGNACGG GTTGNGGTNA	2400
ATNAANNCCC CTCCTTCGTC TATTCANCCC TTCCTATGNA CACTTCCTGN CCCCCTATCT	2460
CTCTATNTNC TNCTCTCTAT ATCTNNATCC CNTCTTCNCN TGCCNCTCCC TNGTNTTNA	2520
NCGGGTATTT NTTNTTCTCC TCNTCTTCTT CCCCTNTNTA NCCNTNCTNC NNNCMNNCCC	2580

## (2) INFORMATION FOR SEQ ID NO:17:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 830 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "5H-1 (Example 10)"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TGGGGGAGAG GACTGAAATA TTTCCACAGC CTTTTTATTG GTGGTGATGG TAGTGATGGT	60
TAGGATTCCT TCTTCTTTC TTTCTTCTT TCTTCTTTC TTTCTTTTTT TTTTTTTTTT	120
TTTTTTTTTT GAGACAGGGT TTCTCTGGGT ACTCCTGGAA CTCACCTTGT GGACCATGAA	180
TGACATGAAT ACTTCGATAT ATACATACAT ACAAAGACAC ATATTTTTTAA AAAGAGAATT	240
AGAGTAGAGC TGGGGCAATT GTGGAACACA CCTTTAACCT CAGGCAGATT TCTGCGTTCA	300
AGGTCACCTT GGATTACAAG GCAGCTAGGG CTACACAGAG AAACCATATC TCAAAAAAAAA	360
GAAAAAATAA TGAAAGAAAG AAAGGAAGGA AGGAAGGAAG GAAGGAAGGA AGGAAGGAAG	420
AAAGGAAGGT AGGAAGAAAG GTATTTTCTT AAAAAAAAAA AAAAAAAAAA TTTATTCCGG	480
GCAGTGGTGG CAAATGCTTT TAATCCCACC ATTTGGGAAA GCAGAGGCAG ACAGATTAAA	540
TTTTCAAGGC CCACCTGGTC CTACACAGTG AATTCCAGGA ACACCTAGGT TTACCCANAA	600
AAAACCCCCC CTTGAAATAA ACAAATAA ATTAATAAAA TAAAATTAA AAATAAAACC	660
CGGGCGTTAA ACCCNCTTTT ATCCCCCAC TTNGGAAGCA AAAGCCGGCN GATTTCTGAA	720
TTCNAGGCCN CCCTGTCTAT GAATTANTTC CCNGAACACC CNAATTTTTC NAAAAACCCC	780
CCNTTCTTA AAAANCCAA ATTATTATTN ATTAATTAAA TNAAATTACC	830

## (2) INFORMATION FOR SEQ ID NO:18:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 838 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "AN8T7 (Example 10)"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GGAGTCCAAC AATGGTTTCC ACTTGTCTGG CGGCCGCTCT AGAGTTTCCC ATAAGCTGGA	60
CTGAGAGATG GTGTGATTGC TGTGGGTGAC AAAGACAGAG GCACCTTTCA TCTCTACCCT	120
TCTCTTGT TTGTTGTTTGT TTGAGACCGG TCCCCACTAT GTAGACCAGG CTGGAGGACA	180
GGGTCTCACT ATGTAGACCA GGCTGGCCTT GAACTCAAAG ACATCTGCCT GCCTCTGCCT	240
CCTGAGGGCT GGGATTAAAG GCGTGTGCTG CCACTGACAG CTTCTATCCT CCTGTCACTA	300
GTCCCGGCTC ACAGGGCCAG AAGATCTCTT CTATGCTTCC ACTATTTCCC CAATCCATTC	360
CCACGGCAGC CTCTCCATCT CCCTACCACC AAGACAGCAG CCTAGTGATA TAACAAAAC	420
TTTATTACACA GGAAACCGGA AAACAAAATC ACAACCAATC ATTTCTATCT AGTCCCTGCC	480
CTAGCCCTCC CTCCAAGCCC CTACATATCC TCCATCTGAG GGGGATGCAT GCGTTGGGTG	540
GGAGCTGCCG GCATCCTTAT CCTGGTTCCT GGAGTAGNGA AGAGTGTTTC TTTTCAACGN	600
CTAGGGNNCT CCCCTCCAAG TTNGGACCTC TCTTCCCAGG NCTTCNCCCC TCCCTNACAG	660
GGNACAAAAA ACCAGGNACG GCACNACGCC AGGNAGGAAG GGA CTCTTG NAATGTTGGG	720
CAGGACTTGT CCTCAGAATT CCNNGGAGGA ATCAAGGGCC TTGAATTCGG GAACCACTNC	780
CGAGGNCTTC ANCANGGCAN AGTTCAATTT TCCATCCCGG TTGGCCANC CTGGCCNG	838

## (2) INFORMATION FOR SEQ ID NO:19:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 180 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "CHINT (TABLE 5)"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TAGTGCCGGT CAAGGAACTG AACGTGCGAT TCCGGGACAG GCTACCCACT CCGATCCCAG	60
GAGAAGTTGT CATGGTGAGG GCCACCCTAG GTCTCTGCCC CTGCTGTGTC CCCCATCTTA	120
CCCATCCAGT AGGATCTAGA GGCTGTGCGC CCCTGTGGA ATGCACAGAA GTCACAAGCG	180

## (2) INFORMATION FOR SEQ ID NO:20:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 175 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "HUMMDB (TABLE 5)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CCTCACCTCC GCCCTGTTTC GTCCAGGTCC TCCGGGTCAG GCTACCCCCG TCGCCGCCAG	60
AGCGCGGGGG AGGGGAGAGC TTCCTTTGTC TCCTATGCCT CCTCCCCCA TCCCGGCTCT	120
CCTGCGGGCA AGCGCCGAGG GGACACCGGG GAGTACCCA CCTGAACCTC TGGGG	175

CLAIMS

What is claimed is:

- 5           1. A method of detecting cancerous cells in a patient by detecting alterations of PP2C $\alpha$  gene activity in a specimen isolated from the patient.
- 10           2. The method of claim 1 wherein the specimen is selected from the group consisting of tissue biopsies and bodily fluids.
- 15           3. The method of claim 1 further characterized by the alteration being a reduction in PP2C $\alpha$  gene activity compared to normal controls.
- 20           4. The method of claim 1 wherein said detecting steps is further defined as assaying the specimen for mRNA complementary to PP2C $\alpha$  DNA including polymorphisms thereof with an assay selected from the group consisting of *in situ* hybridization, Northern blotting and reverse transcriptase - polymerase chain reaction.
- 25           5. The method of claim 1 wherein said detecting step is further defined as assaying the specimen for a PP2C $\alpha$  gene product including polymorphisms and peptide fragments thereof with an assay selected from the group consisting immunohistochemical and immunocytochemical staining, ELISA, RIA, immunoblots, immunoprecipitation, Western blotting, functional assays and protein truncation test.
- 30           6. The method of claim 5 wherein the specimen is bodily fluids selected from the group consisting of urine, blood, cerebrospinal fluid and saliva.
- 35

7. The method of claim 1 wherein the detecting of PP2C $\alpha$  gene activity in a specimen is by determining alterations in phosphorylation patterns of proteins affected by the PP2C $\alpha$  gene product.

5

8. A kit for detecting PP2C $\alpha$  activity as set forth in claim 4, said kit comprising:

a molecular probe complementary to genetic sequences of a mRNA for PP2C $\alpha$  including polymorphisms thereof and

10

detection means for detecting hybridization of said molecular probe and the mRNA thereby indicating the activity of the PP2C $\alpha$  gene.

15

9. A kit for detecting a gene product associated with PP2C gene activity as set forth in claim 5, said kit comprising:

an antibody which with high specificity recognizes markers selected from the group consisting of the PP2C $\alpha$  gene product including polymorphisms thereof and peptide fragments thereof, and

20

detection means for detecting the binding of the antibody thereby indicating the presence of the gene product.

25

10. A kit for detecting a gene product associated with PP2C gene activity as set forth in claim 5, said kit comprising:

an agent which mimics natural proteins which bind to the PP2C $\alpha$  gene product including polymorphisms thereof and peptide fragments thereof, and

30

detection means for detecting the binding of the agent thereby indicating the presence of the gene product.

35

11. A non-human transgenic mammal or cell line containing an expressible nucleic acid sequence for human PP2C $\alpha$  including polymorphisms thereof.

5 12. A non-human eucaryotic organism in which the equivalent genomic nucleic acid sequence for PP2C $\alpha$  is knocked-out.

10 13. A vector comprising an expression control sequence operatively linked to the nucleic acid sequence of PP2C $\alpha$ .

14. A host cell transformed with the vector of claim 13.

15 15. A vector comprising an antisense sequence of PP2C $\alpha$ .

20 16. An antibody which specifically binds to an epitope of a gene product of PP2C $\alpha$  including polymorphisms thereof which distinguishes the gene product of PP2C $\alpha$  from the gene product of PP2C $\beta$ .

25 17. An antibody of claim 16 conjugated to a detectable moiety.

18. An antibody of claim 16 selected from the group consisting of monoclonal and polyclonal antibody.

30 19. A polyclonal antibody of claim 18 raised against recombinantly produced PP2C $\alpha$ .

35 20. A polyclonal antibody of claim 18 raised against the carboxy terminal peptide of pp2c $\alpha$  selected from the group consisting of NDDTDSASTD (SEQ ID No:1) and YKNDDTDSTSTDDMW (SEQ ID No:2).

21. A monoclonal antibody of claim 18 which does not cross-react with pp2c $\beta$  and which is raised against peptides selected from the group consisting of recombinantly produced pp2c $\alpha$ , NDDTDSASTD (SEQ ID No:1) and YKNDDTDSTSTDDMW (SEQ ID No:2).

22. A monoclonal antibody of claim 21 designated as 2A3.

23. An isolated and purified peptide selected from the group consisting of NDDTDSASTD (SEQ ID No:1), YKNDDTDSTSTDDMW (SEQ ID No:2) and PNKDNDGGA (SEQ ID No:3).

24. The peptide of claim 23 produced recombinantly.

25. A method of treating cancer including the steps of

- a. determining the type of cancer and cells expressing the cancer,
- b. preparing a vector which will specifically target the cancer cells including regulatory elements to control the expressibility of PP2C $\alpha$ , and
- c. administering the vector to the patient.

26. The method as set forth in claim 25 wherein the vector includes an AAV modified sequence or part of the AAV sequence.

27. The method as set forth in claim 25 wherein the vector contains the CHINT sequences.

28. The method as set forth in claim 25 wherein the vector includes the silencer region (SEQ ID No:13).

29. The method as set forth in claim 25 wherein the vector includes the mini-silencer region (SEQ ID No:14).

5 30. A method of treating cancer including the steps of

a. determining the type of cancer and cells expressing the cancer,

10 b. preparing an antisense vector which will specifically target the cancer cells to control the expressibility of PP2C $\alpha$ , and

c. administering the vector to the patient.

15 31. A pharmaceutical composition consisting of a vector and a pharmaceutically suitable carrier wherein the vector is selected from the group consisting of a vector which will specifically target the cancer cells and including regulatory elements to control the expressibility of PP2C $\alpha$  and an antisense vector which

20 will specifically target the cancer cells to control the expressibility of PP2C $\alpha$ .

25 32. A method of treating diseases due to aberrant phosphorylation due to alteration of expression of PP2C $\alpha$  including

a. preparing an antisense vector which will specifically target cells expressing aberrant phosphorylation to control the expressibility of PP2C $\alpha$ , and

30 b. administering the vector to the patient.

35 33. A method of suppressing gene amplification by interrupting unscheduled interactions of DNA polymerase  $\alpha$  primase with the gene product of PP2C $\alpha$  by preparing an antisense vector which will specifically target the



binding region of DNA polymerase  $\alpha$  primase to the PP2C $\alpha$  gene product and delivering the vector to the cells.

34. A method for the activation of the gene product of PP2C $\alpha$  expressed on the surface of a cell to induce signal transduction.

35. The method of claim 34 wherein an antibody is used to bind to the gene product of PP2C $\alpha$ .

36. A method of detecting cancer in a patient by detecting altered PP2C $\beta$  gene activity in a specimen isolated from the patient.

37. The method of claim 36 further characterized by detecting an increase in PP2C $\beta$  activity.

38. The method of claim 36 wherein the detecting of PP2C $\beta$  activity is by assaying the specimen for mRNA complementary to PP2C $\beta$  DNA including polymorphisms thereof with an assay selected from the group consisting of *in situ* hybridization, Northern blotting and reverse transcriptase - polymerase chain reaction.

39. The method of claim 36 wherein the detecting of PP2C $\beta$  activity is by assaying the specimen for a PP2C $\beta$  gene product including polymorphisms thereof with an assay selected from the group consisting immunohistochemical and immunocytochemical staining, ELISA, RIA, immunoblots, immunoprecipitation, Western blotting, functional assays and protein truncation test.

40. An antibody which specifically binds to an epitope of a gene product of PP2C $\beta$  including

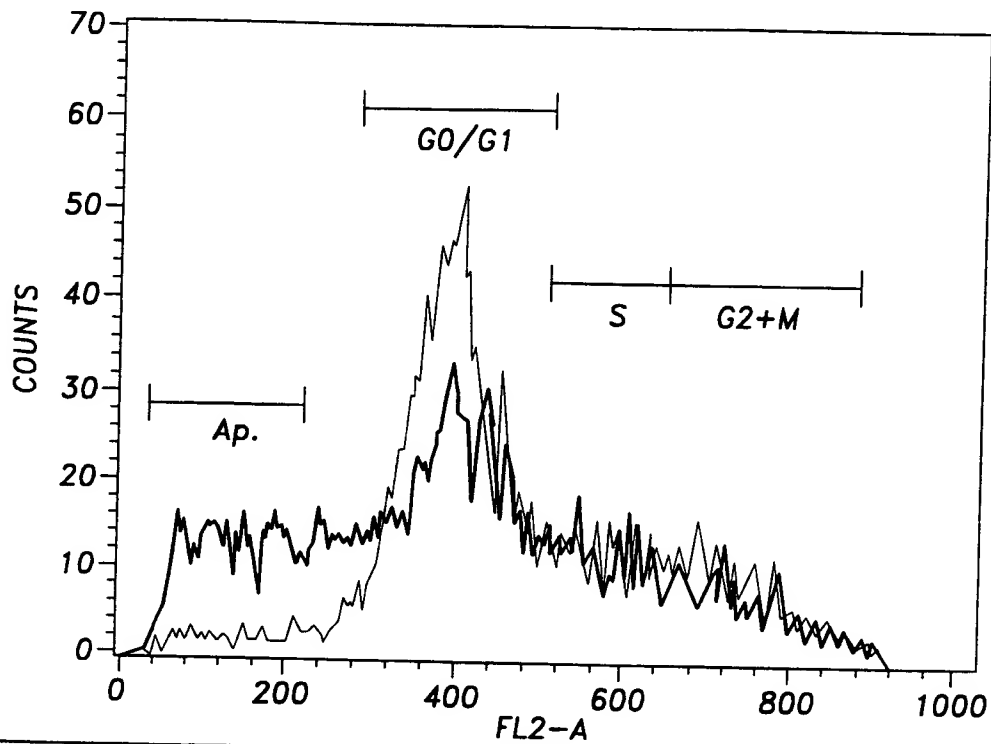
polymorphisms thereof which distinguishes the gene product of PP2C $\alpha$  from the gene product of PP2C $\beta$ .

5        41. An antibody of claim 40 conjugated to a detectable moiety.

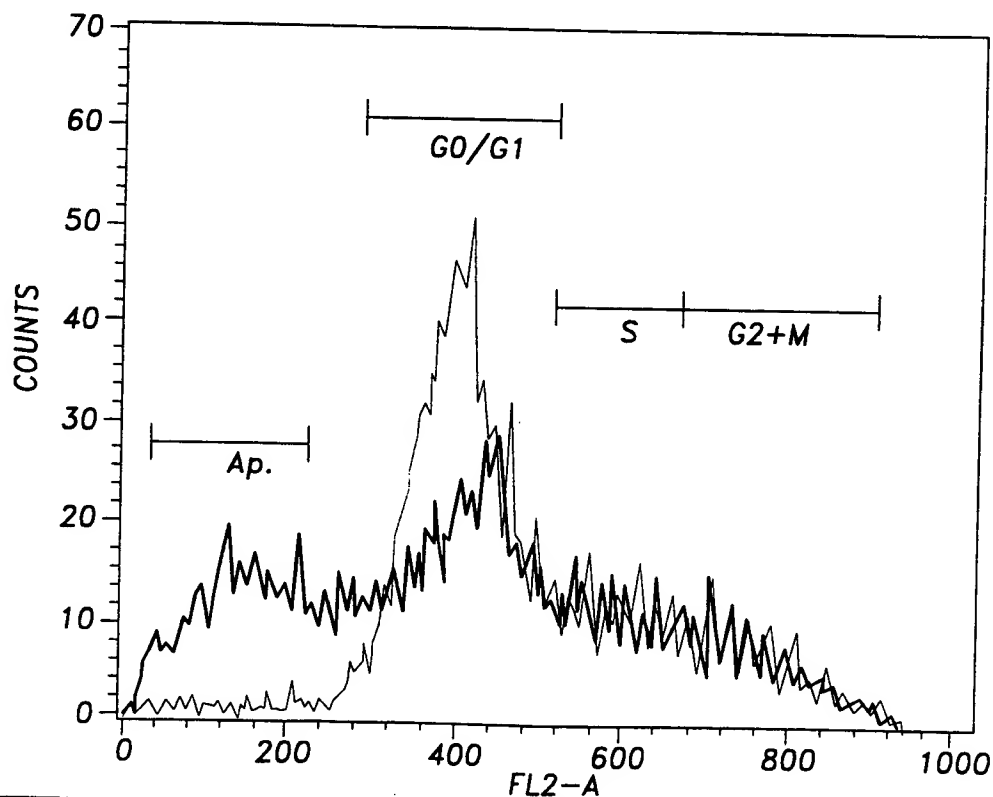
42. An antibody of claim 40 selected from the group consisting of monoclonal and polyclonal antibody.

10       43. A polyclonal antibody of claim 40 raised against recombinantly produced PP2C $\beta$ .

15       44. A polyclonal antibody of claim 40 raised against the carboxy terminal peptide PNKDNDGGA (SEQ ID No:3).



KEY	NAME	PARAMETER	GATE	
—	SHU..005	FL2-A	NO GATE	Co60
—	SHU..008	FL2-A	NO GATE	913

Fig-1a

KEY	NAME	PARAMETER	GATE	
—	SHU..005	FL2-A	NO GATE	Co60
—	SHU..010	FL2-A	NO GATE	916

Fig-1b

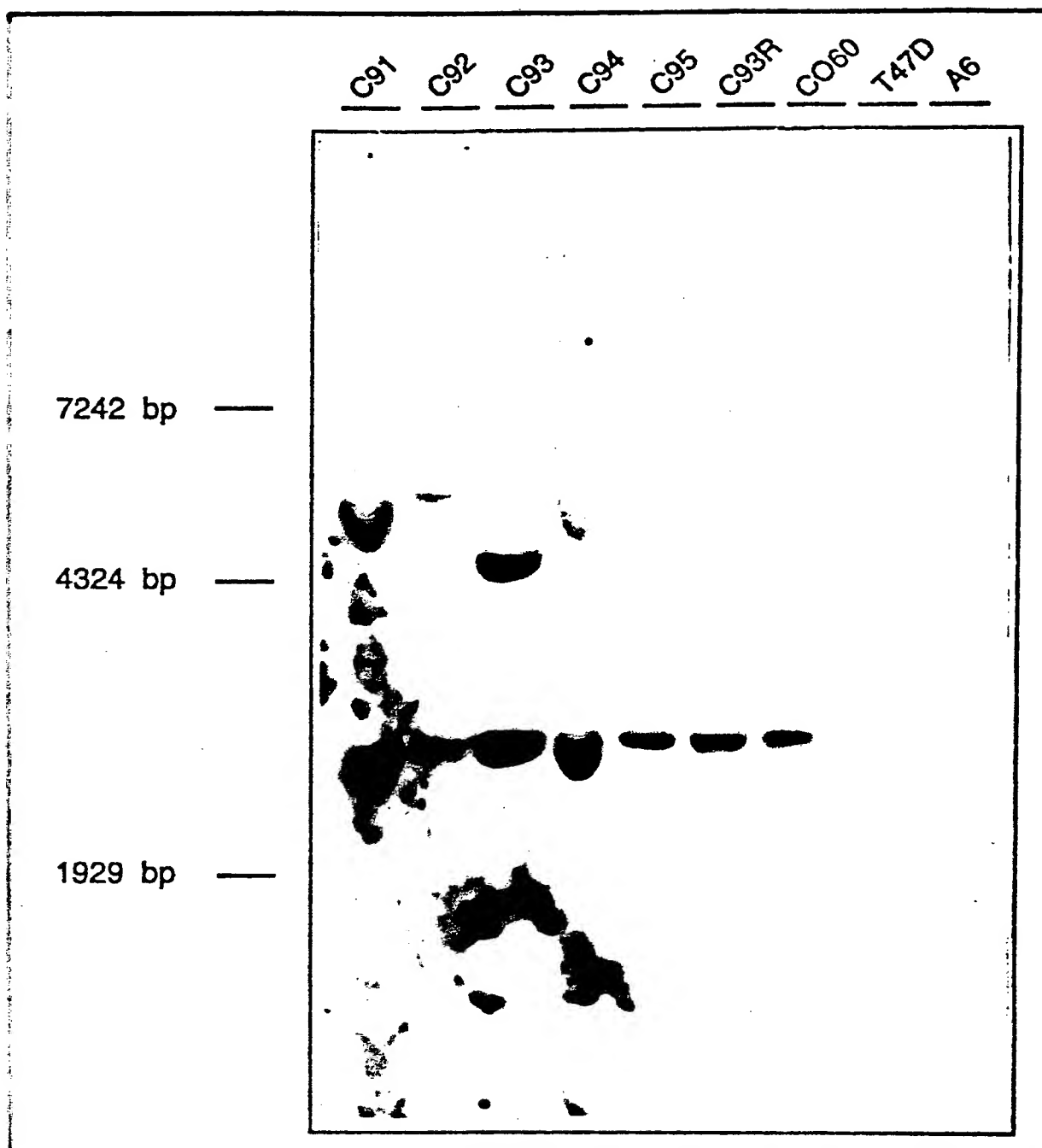
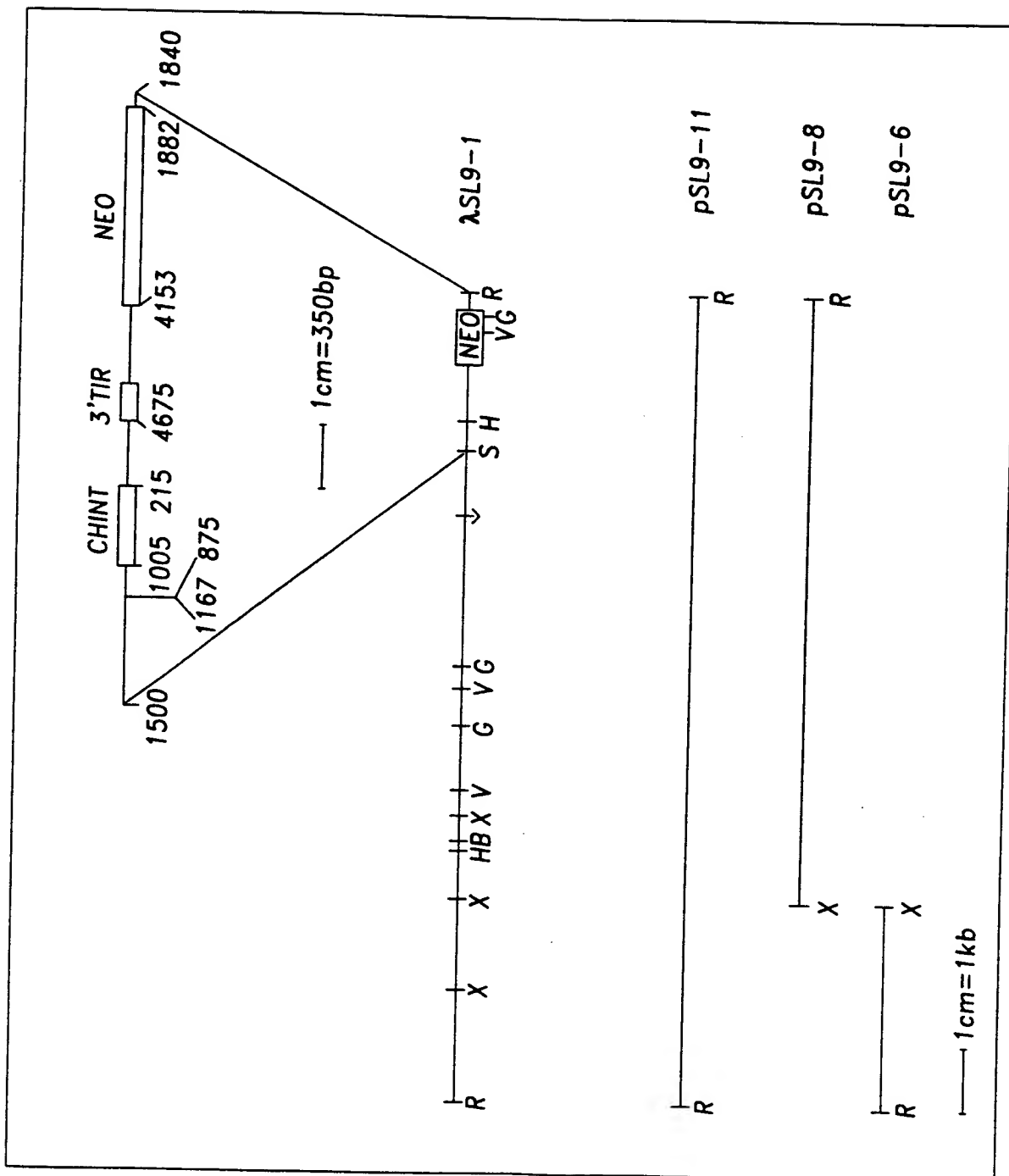
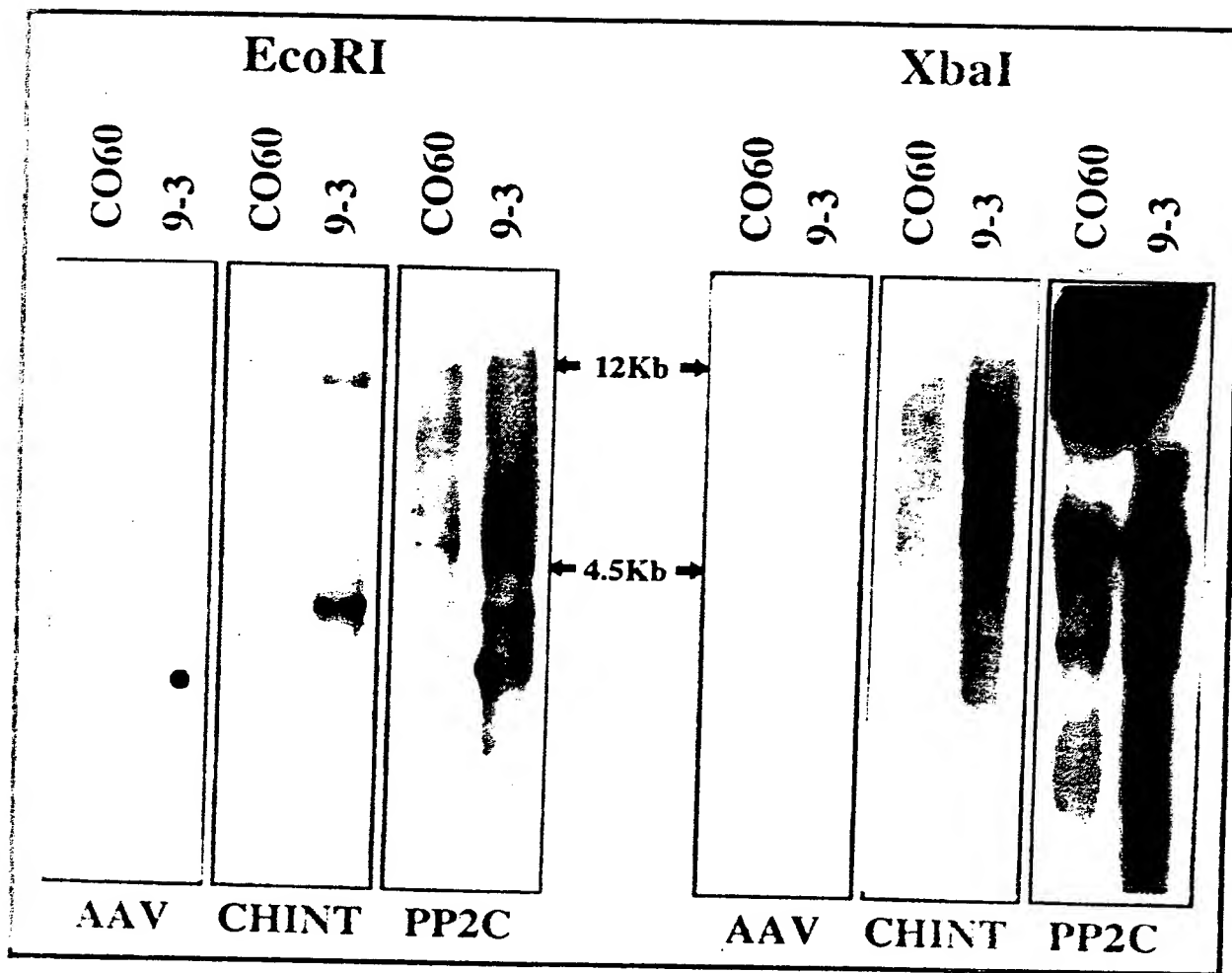


Fig-2

**Fig-3**



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Fig-4a

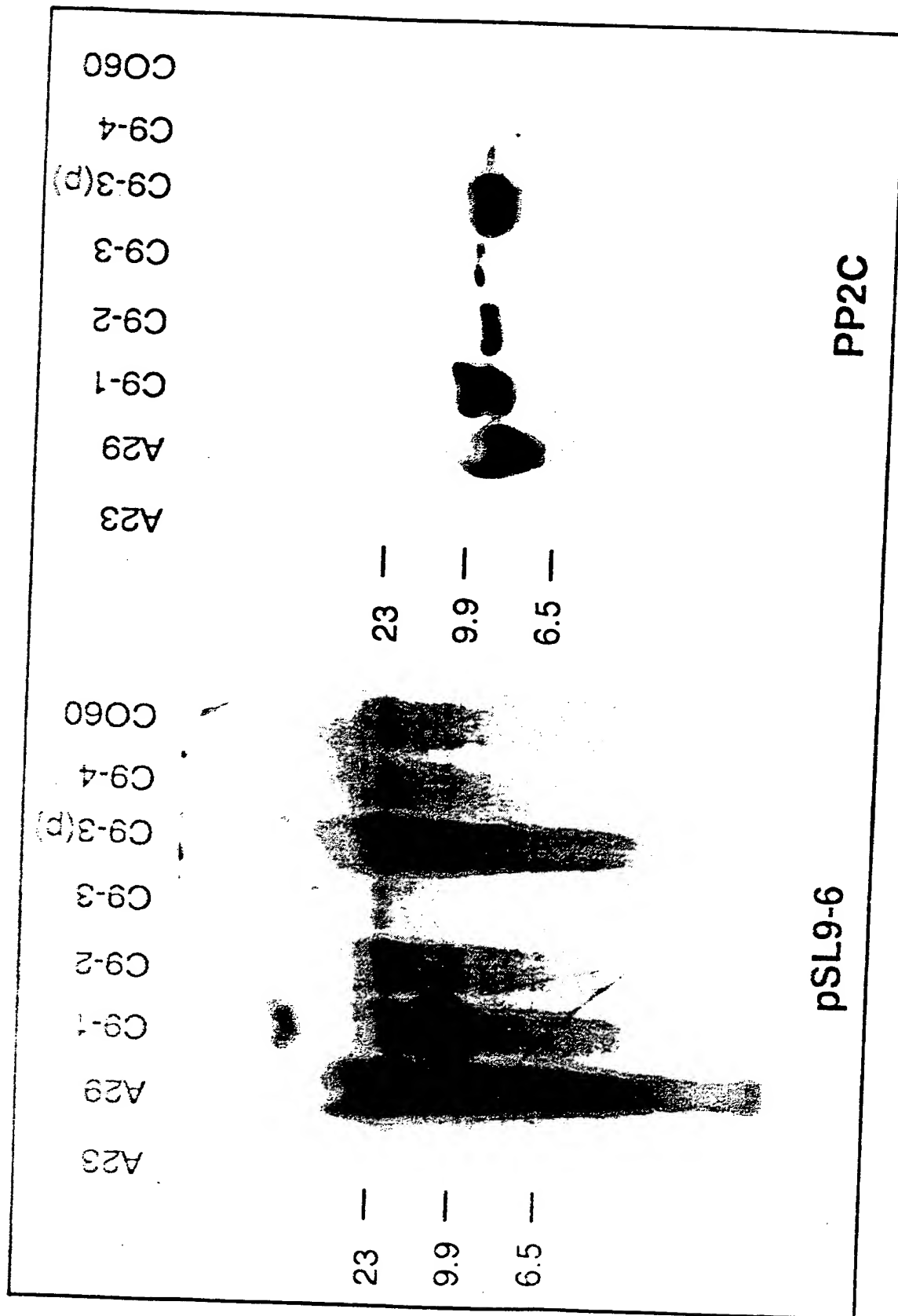


Fig-4b

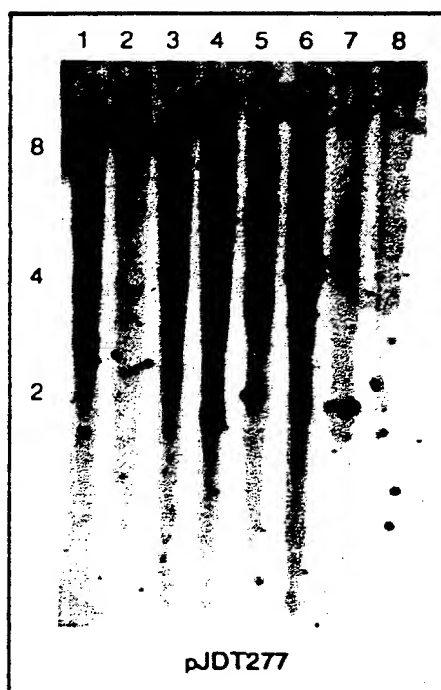


Fig-5a

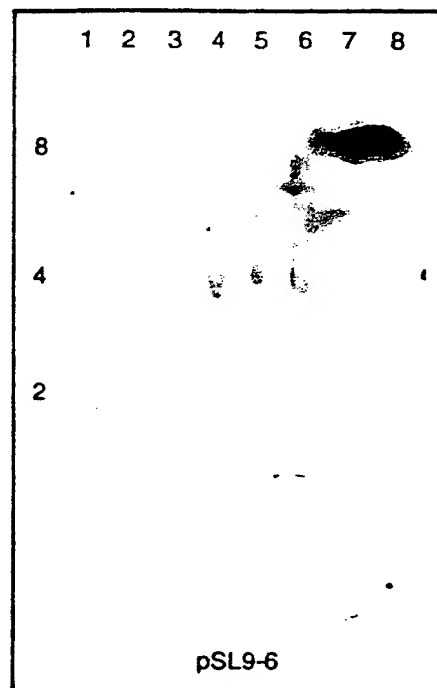


Fig-5b

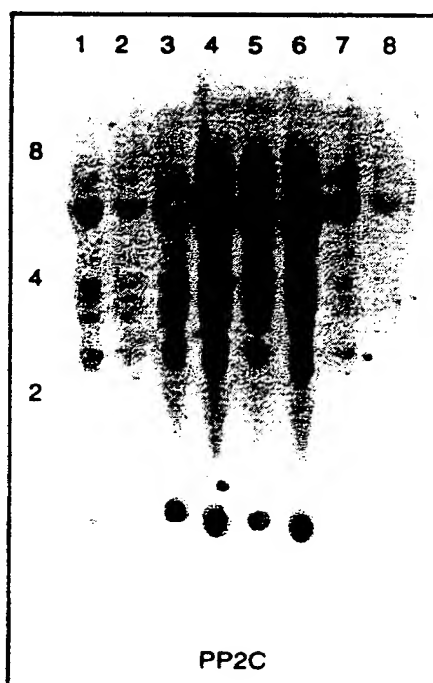


Fig-5c



6/12/1

Fig-6a

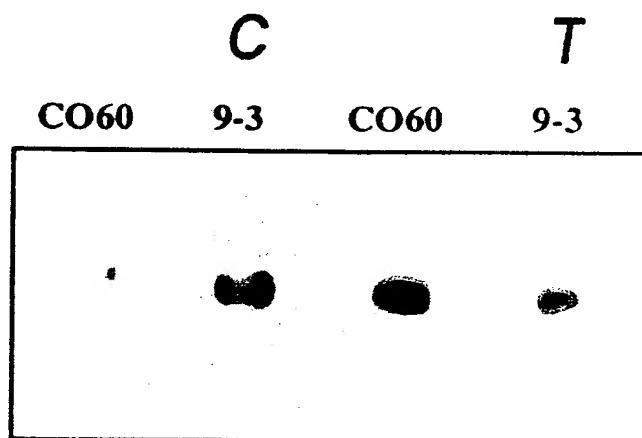
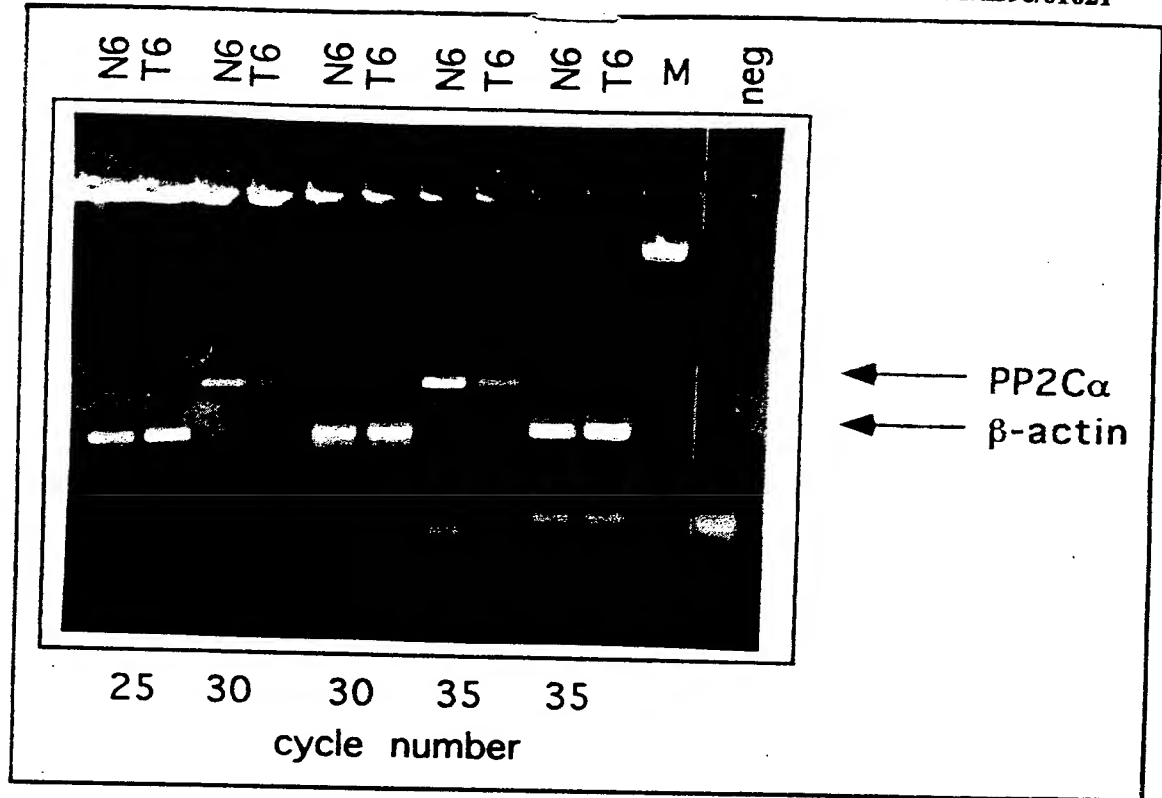
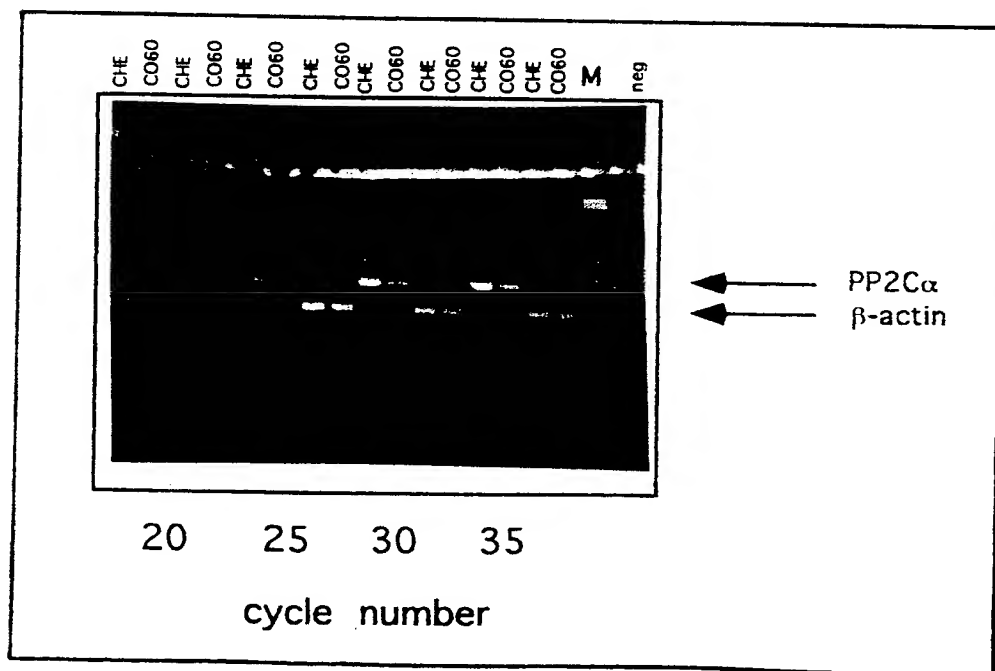
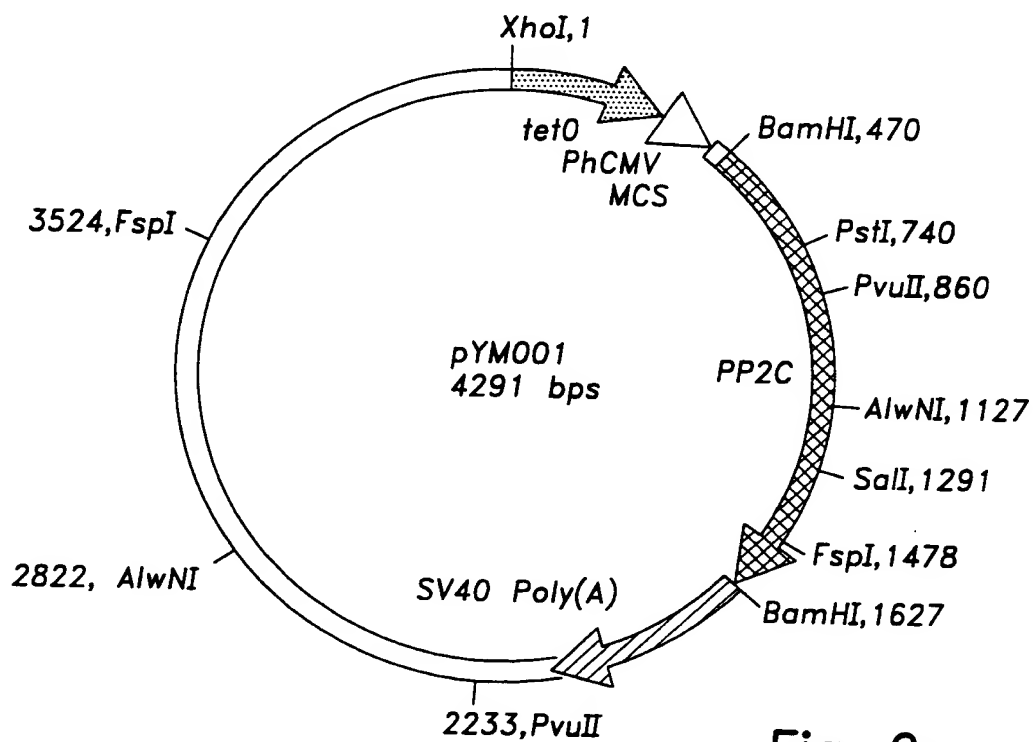
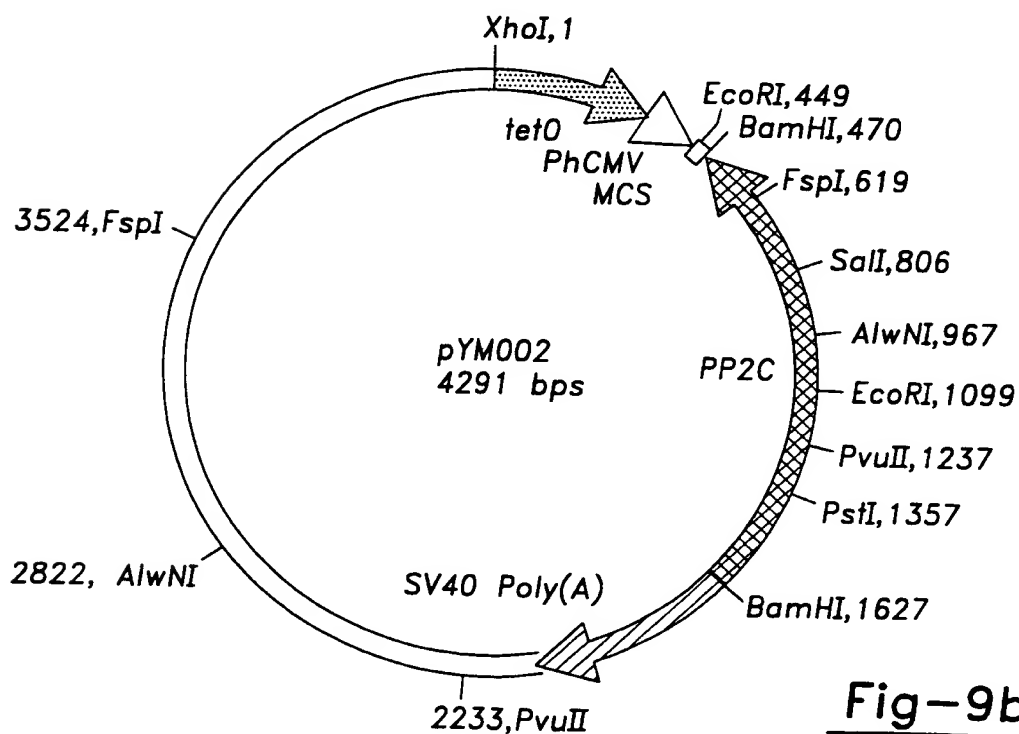


Fig-6b

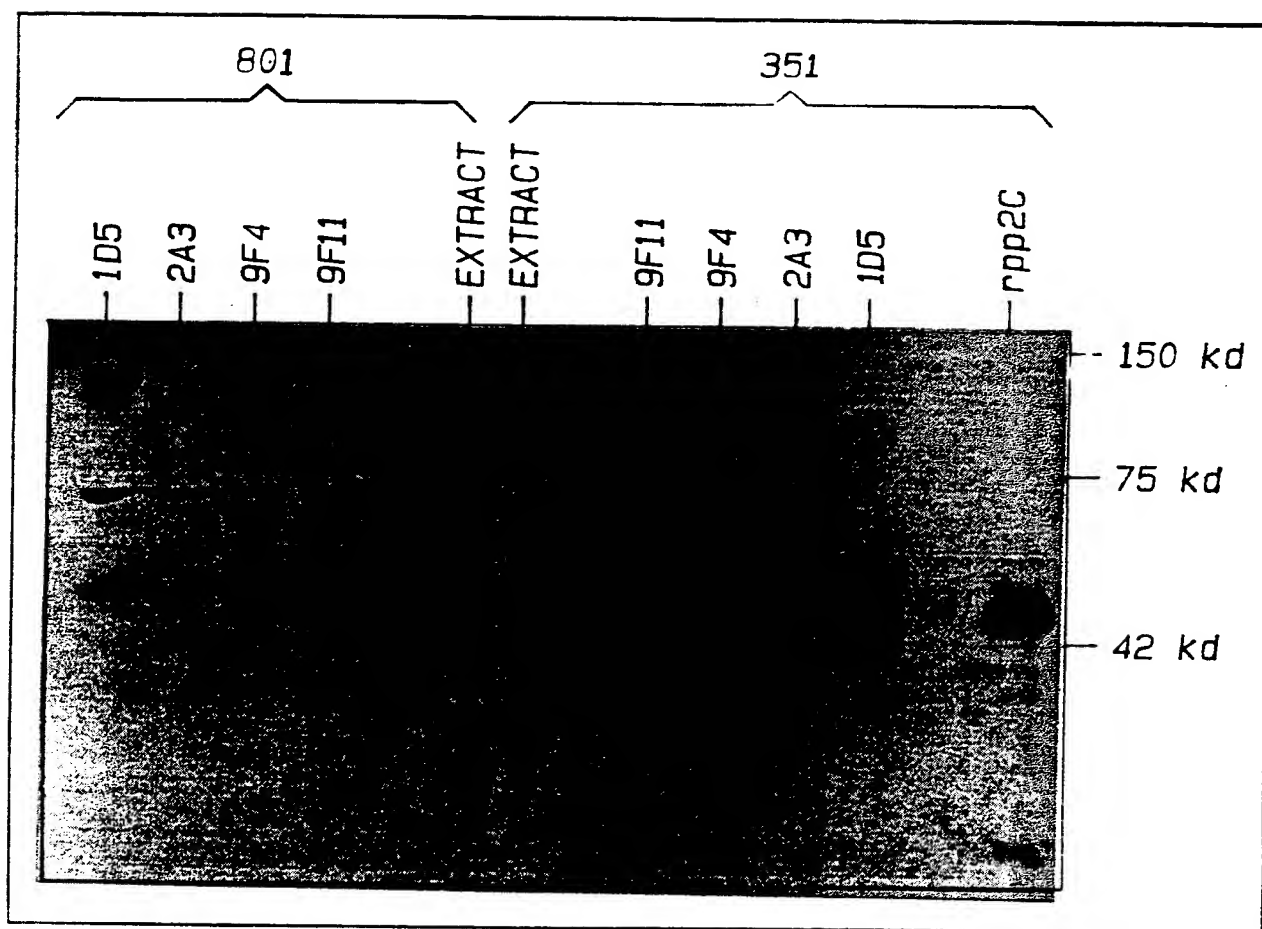


Fig-7Fig-8

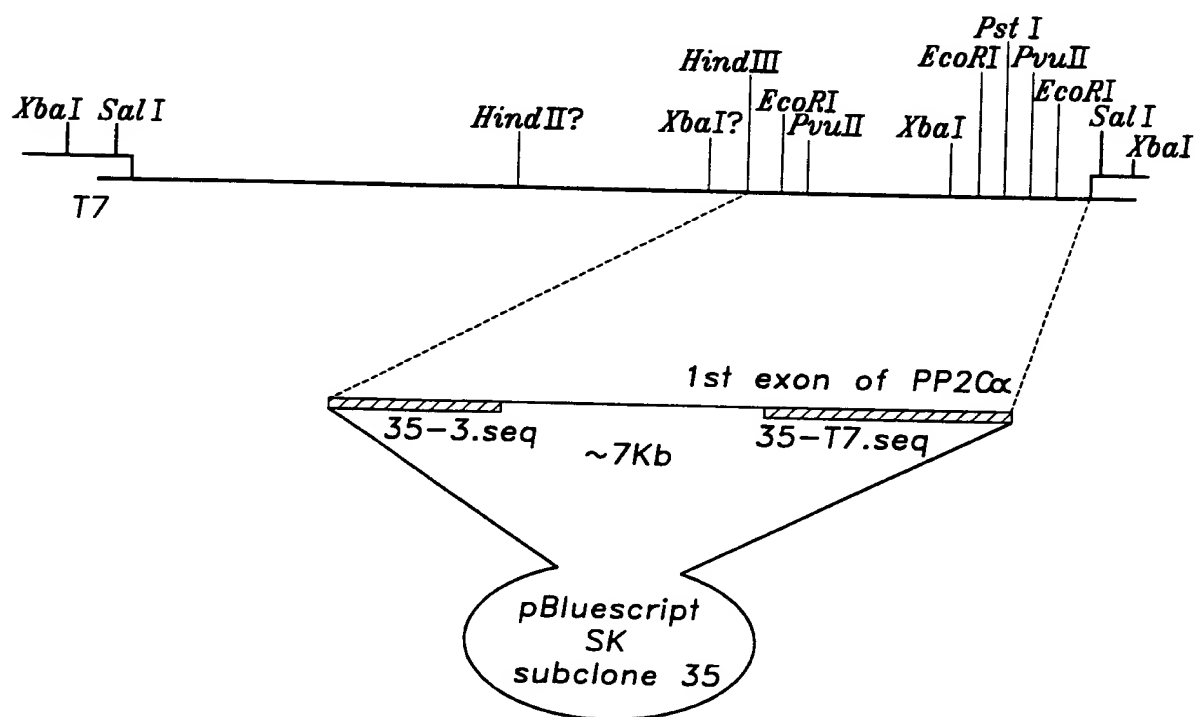
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Fig-9aFig-9b

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Fig-10

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Fig-11

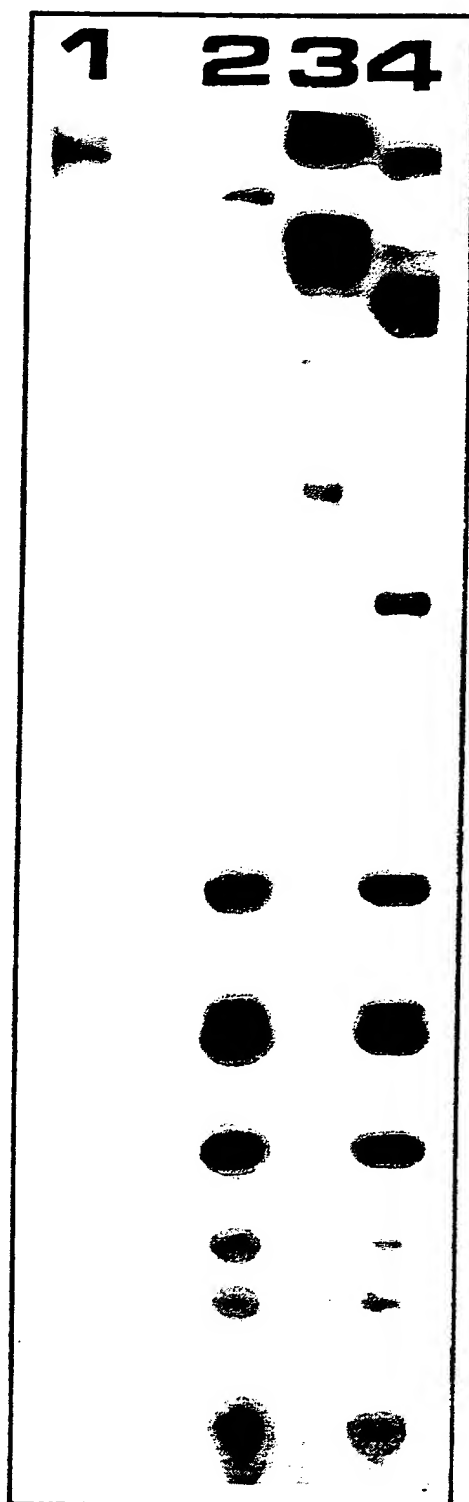


Fig-12

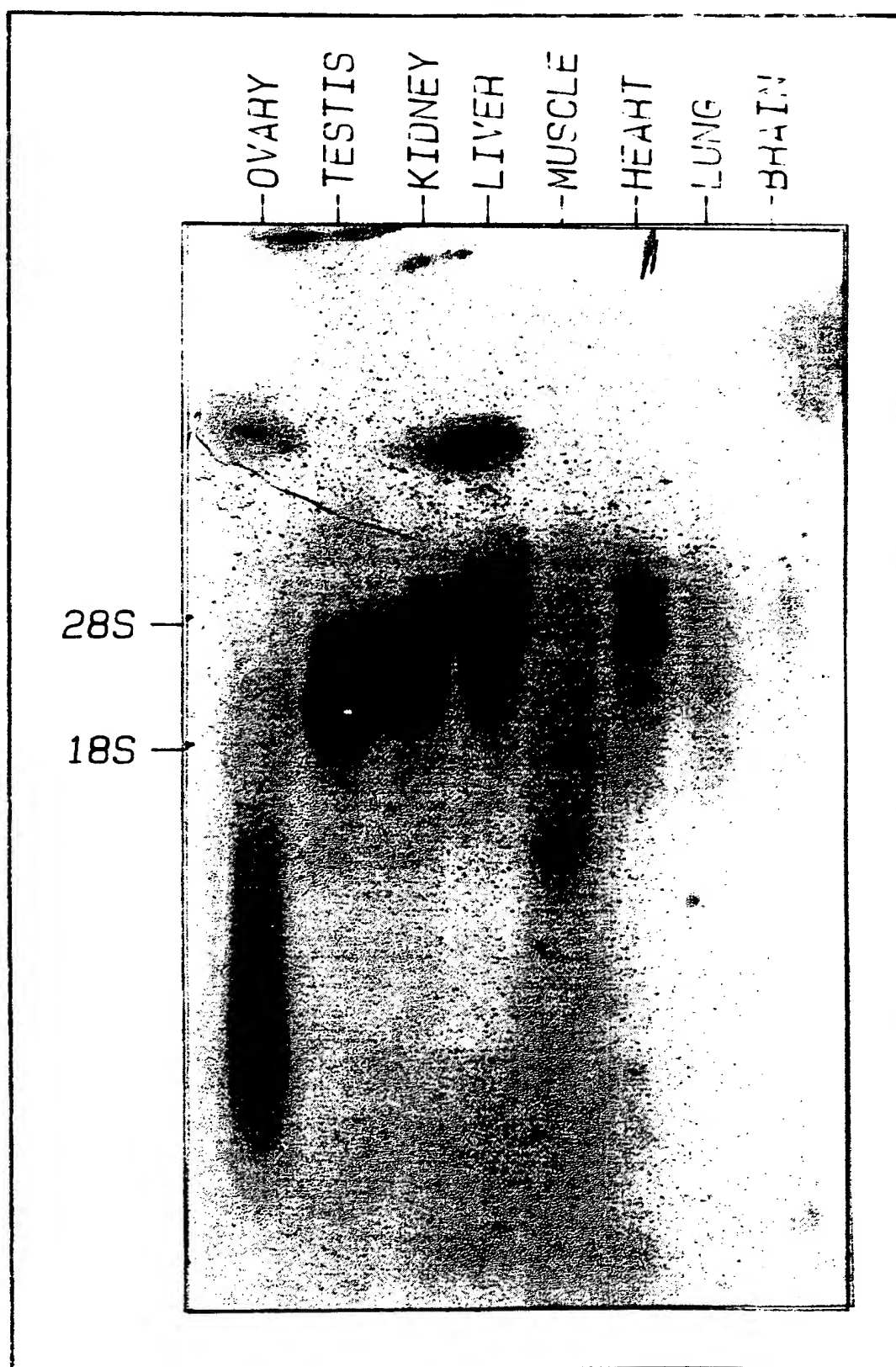


Fig-13

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DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
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<b>(21) International Application Number:</b> PCT/IB96/01021 <b>(22) International Filing Date:</b> 30 August 1996 (30.08.96) <b>(30) Priority Data:</b> 60/003,114 1 September 1995 (01.09.95) US <b>(71) Applicant (for all designated States except US):</b> RAMOT- UNIVERSITY AUTHORITY FOR APPLIED RESEARCH AND INDUSTRIAL DEVELOPMENT LTD. [IL/IL]; 32 Haim Levanon Street, Ramat-Aviv, 61392 Tel-Aviv (IL). <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only):</b> LAVI, Sara [IL/IL]; 6 Tnuat Hameri Street, 55000 Kyriat Ono (IL). <b>(74) Agent:</b> HACKMEY, Michal; A.E. Mulford, 11 Shlom zion Hamalka Street, P.O. Box 544, 91004 Jerusalem (IL).		<b>(81) Designated States:</b> AL, AU, BB, BG, BR, CA, CN, CU, CZ, EE, GE, HU, IL, IS, JP, KP, KR, LK, LR, LT, LV, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, TR, TT, UA, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>  <b>(88) Date of publication of the international search report:</b> 19 June 1997 (19.06.97)
<b>(54) Title:</b> MANIPULATION AND DETECTION OF PROTEIN PHOSPHATASE 2C-PP2Ca - EXPRESSION IN TUMOR CELLS FOR CANCER DETECTION AND THERAPY  <b>(57) Abstract</b>  A method of detecting cancer in a patient by detecting alteration of activity of the gene coding for human type protein phosphatase 2C (PP2Ca and PP2Cβ) and genetic polymorphisms thereof in a specimen isolated from the patient is disclosed. The invention further provides a method of treating cancer including the steps of first determining the type of cancer and cells expressing the cancer and then preparing a vector which will specifically target the cancer cells and can include regulatory elements to control the expressibility of PP2Ca. The vector is then administered to the patient. Alternatively an antisense vector can be prepared.		

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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB96/01021

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 15/63, 5/00, 15/00; A01N 43/04; A61K 31/70

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/172.3, 69.1, 69.2, 325, 7.1, 91.2, 320.1; 530/350, 387.1; 800/2; 536/23.1, 23.5; 424/93.21, 9.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, EMBASE, BIOSIS, CAPLUS, WPIDS, APS  
search terms: protein phosphatase 2C, PP2C, cancer, transgenic

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	SCHAFER et al. Monoclonal anti-FLAG antibodies react with a new isoform of rat Mg <sup>2+</sup> dependent protein phosphatase $\beta$ . Biochemical and Biophysical Research Communications. 15 February 1995, Vol. 207, No. 2, pages 708-714, see entire document.	40-42 ----- 36-39
X --- Y	YOKOYAMA et al. PP2C phosphatase activity is coupled to cAMP-mediated pathway in rat parotid acinar cells. Biochemistry and Molecular Biology International. July 1995, Vol. 36, No. 4, pages 845-853, see entire document.	16-22, 34-35, 40-42 ----- 23-24, 43-44

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
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Facsimile No. (703) 305-3230

Authorized officer

JILL SCHMUCK

Telephone No. (703) 308-0196

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## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y --- Y	SAADAT et al. Gene expression of protein phosphatases in rat ascites hepatoma cell lines. Cancer Detection and Prevention. 1994, Vol. 18, No. 2, pages 115-122, see entire document.	1-10, 13-15, 23-33
Y	KLUMPP et al. A membrane-bound protein phosphatase type 2C from <i>Paramecium tetraurelia</i> . Purification, Characterization, and Cloning. The Journal of Biological Chemistry. 30 December 1994, Vol. 269, No. 52, pages 32774-32780, see entire document.	13-15, 23, 24

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US CL :

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